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Evaluation of the efficacy of an augmentation material for peri-implant bone diseases in minipigs

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Zusammenfassung

Peri-Implantitis ist eine Komplikation von osseointegrierten Zahnimplantaten mit Knochenresorption und Weichteilschäden. Die Stabilität von Implantaten kann durch Augmentation erhalten werden. In dieser Studie wurde die Effektivität der Augmentation mit einem Hyaluronsäure-Gel mit Hydroxyapatit-Nanopartikeln (Test Item (TI) 1) oder zusätzlich Bisphosphonaten (TI2) getestet. Die Studie bestand aus 2 Teilen mit je 4 Minipigs (n=8). 4 Standard Zahnimplantate wurden auf jede Seite der Mandibeln implantiert. In Part A (A) wurden akute, bukkale Defekte kreiert und in Part B (B) wurde eine Peri-Implantitis mit Fäden induziert. Die Defekte der einen Seite wurden mit TI1 oder TI2 gefüllt und die der anderen wurden als Kontrolle (Ctr) leer gelassen. Die Tiere wurden 6 (A) oder 4 (B) Wochen nach der Implantation des TI getötet. Alle Implantate zeigten eine hochgradige Peri-Implantitis mit erodierten Implantaten. CT Messungen zeigten weiteren Knochenverlust. Histologisch konnte in keiner evaluierten Probe mineralisiertes Gewebe oder Reste des TI nachgewiesen werden. Die Ergebnisse von TI und Ctr Implantaten sind ähnlich, mit einem Trend für bessere Ergebnisse des TI2. Viele unbekannte Faktoren und Komplikationen machen eine klare Aussage zu der Effektivität und der Entwicklung des Gels *in vivo* unmöglich. Das gewählte Tiermodell konnte nicht zwischen Ctr und TI unterscheiden. Für künftige Studien muss ein besseres Tiermodell gefunden werden um die Effektivität des Gels zu untersuchen.

Stichworte: Peri-implantitis, Hydroxyapatit, Bisphosphonate, Knochen Augmentation, Tiermodell, Hydrogel, Zahnimplantate

Summary

Peri-implantitis is a complication of dental implants resulting in bone resorption and soft tissue damage. To maintain implant stability dismantled bone might be augmented. In this study, the efficacy of the augmentation with a hyaluronic acid gel containing either only nano-particles of hydroxyapatite (Test Item (TI) 1) or additional bisphosphonates (TI 2) was investigated. The study consisted of 2 parts including 4 minipigs each (n=8). 4 standard dental implants were placed on each side of the mandible. Acute buccal defects were created in Part A (A) and a ligature induced peri-implantitis was provoked in Part B (B). Defects of one hemimandible were filled either with TI 1 or TI 2, the defects on the other side were left empty as a control (Ctr). Animals were sacrificed 6 (A) or 4 (B) weeks after TI implantation. In all implant locations, a severe peri-implantitis with eroded implants was observed. CT measurements revealed further bone loss. Histologically, representative samples of Part A and all Part B samples showed no signs of mineralized tissue or remnants of the TI. Results of TI and Ctr sites were similar, with a trend of better results in TI2. But, many unknown factors and complications made a clear statement on the efficacy and the performance of the gel *in vivo* impossible. The animal was not able to differentiate between Ctr and TI. For future studies, an improved and reliable animal model has to be established to evaluate the efficacy of the gel.

Keywords: Peri-implantitis, hydroxyapatite, bisphosphonates, bone augmentation, animal model, hydrogel, dental implants

1 Introduction

1.1 Peri-implantitis

1.1.1 Definition and history

The development of successfully replacing missing teeth with metal implants can be seen as a start of a new era in dental industry. Implantation became a standard procedure over the last decades with around two million implant placements each year [1]. But with this success, peri-implant complications emerged simultaneously: peri-mucositis and peri-implantitis.

An inflammation in the area around dental implants was first described in the 1960s [2]. But it took some decades until the First European Workshop on Periodontology in 1993, to describe peri-implantitis: “inflammatory reactions with loss of supporting bone in the tissues surrounding a functioning implant” [3]. Peri-mucositis on the other hand, is a reversible inflammation of the implant surrounding soft tissue only. It was then at the Sixth European Workshop of Periodontology that the definition was expanded and both diseases were categorised as infectious diseases [4]. Peri-mucositis is associated with redness and swelling of the soft tissue and bleeding on probing. This is seen in peri-implantitis as well but going along with pocket formation, potentially suppuration and always with bone loss. If only bleeding on probing, loss of supporting bone or increased peri implant probing depth is present, it is not sufficient to refer to that as peri-implantitis [5]. It is also important to note that non-osseointegrated implants lost within the first year are not categorised as peri-implantitis [6]. The end stage is the same in both: implant failure.

There have been different attempts for a systematic classification of peri-implantitis. Froum and Rosen (2012) tried to establish a classification with probing depth and bone loss as criteria. They graded three stages [7]:

	Bone loss	Probing depth
Early	<25%	4-6mm
Moderate	25-50%	6-8mm
Severe	>50%	>8mm

Tab 1.1: Classification of peri-implantitis after Froum and Rosen

But until now there is no consensus report about the classification and no standard could be established even though there is a clear need for an accepted classification, not only for clinicians, but also in science. Hence, case definitions differ from study to study because of a lack of standards [8]. As long as there is no accepted classification, the comparison of studies will remain a problem.

1.1.2 Epidemiology

In dental medicine the replacement of teeth with dental implants became a routine procedure during the last decades. With rising numbers of placed dental implants, the total number of infected implants rises alongside.

Mombelli *et al*. evaluated 29 articles and came to the conclusion that around 10% of the implants undergo peri-implantitis in 5-10 years [5]. In the same time around 20% of patients suffer from peri-implantitis. But depending on the case definition, the numbers might be overestimated or underestimated by authors and can vary from 9 % up to 56% [8, 9]. Zitzmann *et al*. include peri-implant inflammations without pocket formation in their survey, whereas Ferreira *et al*. only included peri-implant bone lesions with pocket depths greater than 4 mm. Investigating current literature on the prevalence and incidence of peri-implantitis, Derks *et al*. calculated a mean prevalence of 22% of developing peri-implantitis [10]. The reason for such a large variation is seen in the absence of a clear case definition of the disease. Even in 2014, there was still a lack of consensus in the common literature. In order to compare studies, it is necessary that all investigators apply the same definitions [11].

However, it is not only a lack of definition that makes investigations difficult. Also, many risk factors play a key role in the development of peri-implantitis and they can be responsible for bias in clinical research. Risk factors increasing the incidence of peri-implantitis include smoking tobacco, poor oral hygiene, systemic diseases (e.g. diabetes

mellitus) and a history of periodontitis [9, 12-14]. According to Clementini *et al*. smoking tobacco could be identified as being the biggest influence on the health of implants [15].

1.1.3 Etiology and pathogenesis

Peri-implant diseases begin when osseointegrated dental implants and the surrounding tissue face a bacterial load that does not match the defence barrier [8]. The oral cavity hosts many different kinds of bacteria and bacteria will be found also in healthy teeth and implants as well as inflamed ones. Cleaning mechanisms like swallowing, oral hygiene, epithelium turnover or constant production of saliva are big challenges for pathogens and represent the most important defence barrier of the oral cavity [16]. Nevertheless, teeth and implants are possible targets for pathogens to attach. Fürst *et al*. took samples of implants and surrounding teeth at implantation and found that colonization of bacteria already occurs only 30 minutes post implantation [17]. Hence, the question arises which of the bacteria would provoke peri-implantitis? According to Charalambakis *et al*., Gram positive bacteria are associated with healthy teeth and healthy dental implants [18]. On the other side, during the development of peri-implantitis there seems to be a shift towards gram negative, anaerobic bacteria. *Bacteroides, Fusobacterium nucleatum, Porphyromonas gingivalis, Tannerella forsythia* were detected at higher levels in patients suffering from peri-implantitis [19].

Plaque formation plays the key role in the development of peri-implant diseases. Plaque formation on dental implants occurs when bacteria, cell debris and rests of food attach to the implant surface. Plaques can be expected to develop within three weeks after implantation [20]. Enclosed in the submucosa, bacteria are well protected against mechanical and immune mediated destruction.

The inflammation is characterised by several steps. First, mucosa and gingiva are affected due to an immune response triggered by bacterial colonization. This will result in a peri-mucositis, which progresses due to endogenous inflammatory reactions. Immune cells are the main factor in the destruction, not bacteria and their by-products as itself [6]. If not treated, the infection will invade deeper into the soft tissue and will result in bone resorption at some point. Peri-implantitis lesions are characterised by a high number of polymorphonuclear cells (mostly neutrophilic granulocytes), lymphocytes and plasma cells [21]. Later, epithelial cells will grow into the pockets as well as soft tissue, resulting in a further loss of implant stability. Bone is resorbed by emerging osteoclasts. If not treated appropriately, the inflammation will progress and complete implant loss might by

the consequence. In ligature-induced peri-implantitis animal models, the bone resorption tends not to progress after ligature removal, whereas bone loss proceeds in naturally occurring peri-implantitis until complete implant failure [6, 22].

1.1.4 Prevention

Peri-implantitis treatment can be successful and progression may be limited, even though the predictability varies between the studies [23]. Nevertheless, the best treatment strategy is the prevention of the progression of peri-mucositis to peri-implantitis [24].

Since smoking is seen as one of the biggest factors in the development of peri-implant diseases, it is essential that patients undergo a smoking weaning before the implant placement procedure. Unfortunately, due to the delayed onset of peri-implantitis, patients may not take the additional risk reasonable [12].

Furthermore, in patients suffering from periodontitis, proper plaque control is needed to reduce the risk of developing peri-implant diseases [25]. Also, systemic diseases influence the immune response. Therefore, it is important that systemic diseases, most importantly diabetes mellitus, are treated accordingly for minimizing the effects [26].

Instructed oral hygiene by professionals is seen essential and daily rinsing with antibacterial agents can also be supportive [6, 27].

The surface of implants correlates with speed and quantity of plaque formation. A better osseointegration was achieved with a rough implant surface (Sa (arithmetical mean height) > 2) but less plaque formation is seen on implants with smooth surfaces (Sa < 1) [16]. Nowadays most implant surfaces have a medium roughness (Sa 1-2) [28].

It was shown, that treating peri-mucositis consequently is a major factor in preventing peri-implantitis [24]. It is very important that implants are checked routinely. The number of annual recalls depends on the susceptibility of developing peri-implantitis in each patient and is strongly related to the patient's compliance [29].

Further treatment strategies also strongly depend on the patient's compliance. The challenge for the patient is to keep the mouth as clean as possible. Therefore, authors strongly recommend professional instructions for oral hygiene [29]. Patients should also be enlightened about the importance of following these instructions. Rinsing with antibacterial liquids such as chlorhexidine also seems helpful during the treatment of peri-implantitis [30].

1.1.5 Diagnosis

During recent years many researchers focused on diagnostic possibilities especially within the early phases of peri-implantitis. An early diagnosis and an immediately starting treatment have a strong correlation on the outcome.

But the characteristics are still crucial:

- Bleeding/suppuration on probing
- Pocket formation
- Bone loss

The surrounding tissue of implants should be checked for bleeding or suppuration upon gentle irritation with a blunt instrument. The force with which probing is done should be around 0,25 N [31]. Infected tissue is easy to penetrate in contrast to healthy tissue [6]. Commercial tools with a standardised force are available.

Secondly, the pocket formation around the implants can be identified using an exploring device with a measurement scale on it. Since there is a lack of consensus in literature, the depth can vary from 0 to 6 mm [32].

The assessment of the bone loss is usually done using x-rays. X-rays of peri-implantitis will show a decrease in the bone crest compared to neighbouring teeth. It is important to have post-implantation x-rays as a baseline for comparison with follow-up pictures [29]. For the planning of surgeries for implantation and evaluation of peri-implantitis, a CT scan might be helpful as well.

The idea to detect biomarkers and enzymes in peri-implant cervicular fluid, that can be connected to peri-implant diseases, has not succeeded yet. Some groups could link enzymes and biomarkers to infected implants. But they could not define one with high specificity and sensitivity [33].

1.2 Treatment strategies

Treatment strategies aim for two outcomes: implant stability and reduction of the bacterial load. Thus, debridement of the surface and the surrounding tissue is something all treatments have in common [23]. Since peri-implantitis is a progressive continuing disease, it will end in complete implant loss, if not treated [6]. Because there is no standard treatment protocol developed yet, strategies vary between clinicians [34]. In general, dentists have the possibility to decide between two treatment strategies – nonsurgical and surgical. Which one is chosen depends on the severity of the peri-implantitis. But if bone loss is > 2/3 of the implant length, an extraction of the implant should be considered [29].

The benefit of systemically administered antibiotics as a supportive treatment is controversial [35-37]. There have been favourable results in various studies, but the problem is that control studies are missing [35]. If administered, Rams *et al*. suggest to run out an antibiogram first, since pathogens can be resistant to amoxicillin or metronidazole, but rarely to both [38]. In the after-care, instructed oral hygiene is very important and rinsing with antibacterial liquids seem beneficial [29, 30]

1.2.1 Non-surgical treatment

In this non-invasive approach, the surface of implants and the structures not being covered by mucosa, are cleaned only. The aim of this debridement is to remove plaque and reduce the bacterial load. Many tools are available for doing so, including curettes, ultrasonic devices, air-abrasives and laser. These tools can be used alone or in combination with antimicrobial substances such as chlorhexidine or sodium hypochlorite [23]. Especially in early stages of peri-implantitis, nonsurgical treatments might be effective [39]. In contrast, Renvert *et al*. compared 24 studies and concluded, that nonsurgical treatment strategies are not effective to treat peri-implantitis. But at the same time, following their investigations, nonsurgical treatment is likely to be effective in treatment of peri-mucositis [40]. There is none that is statistically superior to others. Air abrasive devices seem to be the better option compared to mechanical cleaning with curettes [41].
In a single-blinded randomized study, Persson et al. compared the bacteria reduction using curettes and ultrasonic devices [42]. Both methods were neither able to eliminate nor to reduce bacteria in peri-implantitis. Renvert and his group came to the same conclusion in a double blinded study [43].
In most cases a non-surgical treatment will not be enough to treat the inflammation and surgical interventions resulted in a higher decrease of pocket depths [44].

1.2.2 Surgical treatment

Being the more severe disease, a surgical approach is preferred in most cases of peri-implantitis. Parallel to nonsurgical strategies, debridement and reduction of the bacterial load are the main goals. The same devices are used for debridement in surgical approaches and can be combined with antimicrobial substances as well. Since implants are already osseointegrated, smoothing exposed part of the implant surface will make it more difficult for bacteria to attach to it and therefore, it is a possibility to reduce bacterial adhesion.

And especially in severe peri-implantitis situations, bone augmentation may be installed and treated sites might be covered with a guarding membrane [23].

If open surgery is carried out, a mucoperiosteal flap is the access of choice [45]. Flaps are elevated on the buccal and lingual side. This allows a better view on the implant and at the same time more working space. The flap is closed with single stiches or vertical mattress sutures [46]. Suture material can be resorbable or non-resorbable [47]. Which one is used, is the surgeon's choice. The advantage of the non-resorbable one is that patients have to visit for a follow-up treatment.

Mombelli *et al*. compared 33 articles regarding treatment protocols and their outcomes. Unfortunately, no treatment that could act as a gold standard could be identified [45]. But, there may be treatment strategies that are superior. Heitz-Mayfield *et al*. reviewed 43 publications. According to their findings, a staged treatment leads to the best outcome. In an initial phase, oral hygiene should be instructed by a professional and smoking should be reduced, if possible. In a second stage, debridement should be carried out and implant surface can be adjusted. It could be beneficial to stabilize the defects using augmentation material. A covering membrane is optional. It also seems to be beneficial to regularly clean the surface of the implant during recall sessions [23].

Carral *et al*. investigated the effects of mechanical cleaning with a titanium brush, with and without sodium hydrochlorite on one side. They compared the results to a treatment with ultrasonic devices in combination with chlorhexidine. None of the used methods could be detected superior compared to each other. Compared to the control group, they all showed better outcomes [30].

1.3 Augmentation procedures

Especially in cases with a severe bone loss, it can be a great challenge to achieve implant stability. Thereby, bone augmentation might be required. Several defect filling materials have been developed. Augmentation materials might also be combined with barrier membranes to improve chances of success. A strategy which is referred to as guided bone regeneration.

1.3.1 Membrane

Guided bone regeneration describes the usage of a submucosal membrane with or without an augmentation material. Membranes are fixed on top of the eventually filled defect by pinning or with stiches and the flap is closed on top of the membrane.

The membrane works as a barrier between the soft tissue and the bone. Soft tissue usually grows faster than bone regenerates (especially the periodontal ligament). Without a membrane, the soft tissue might invade the area between bone and implant and will reduce the amount of newly built bone [48]. Larrson *et al*. drew a smaller circle with the epithelium being the only tissue that has to be prevented from invading the area of potential bone regeneration [49].

Membranes can be separated into two groups - degradable and non-degradable. The non-degradable material always needs a second surgery to remove the foreign material again. On the other hand, collagen membranes are totally degraded after 5 months [50].

Membranes can be grouped according to the origin of their material as well. Membranes that are used the most are either xenogenic collagen or synthetic expanded polytetrafluoroethylene (ePTFE) membranes. Collagen membranes are mostly made from porcine or bovine type I and III collagen [51, 52]. Since collagen is degraded fast, it can be cross-linked with several chemicals, such as formalin [53]. The downside of collagen membranes is the fast degradation of the material [54]. Clinically, Nociti *et al*. could not find any differences in clinical outcome between ePTFE and collagen membranes[55]. Nevertheless, both membranes have their advantages and disadvantages mentioned in the literature [56]:

	Collagen	ePTFE
Advantages	Biodegradable – no need for second surgery	Not biodegradable – long lasting
	Good tissue integration	Easier to shape
	Fast vascularisation	Increased mechanical integrity
Disadantages	Fast biodegradation	Second surgery
	Resorption may interfere with wound healing/bone formation	Fast colonization with bacteria
	Lack of stability	Soft tissue complications

Tab. 1.1 Table compares the advantages and disadvantages of a resorbable collagen membrane and a non-resorbable expanded polytetrafluoroethylene membrane (ePTFE)

Not only the advantages summarised in Tab 1.1 are responsible for the popularity of membranes. Additionally, membranes have the property to be loaded with drugs, such as antimicrobials or osseoinductive materials, which can be released over time [57].
Using a barrier membrane became a standard procedure in peri-implantitis treatment. In a study by Sculean *et al*., it was stated that around 80% of surgeons use a barrier membrane and most of them have favourable results [50]. Even though using a membrane seems to be beneficial, Roos-Jansaker *et al*. could not prove the improved outcome statistically, when using a barrier membrane [58].

1.3.2 Augmentation materials

In moderate cases of peri-implantation, a resective therapy without augmentation might still be successful. But especially in cases with a severe bone loss, it is mandatory to augment the defects for successful restoration of the bone. The definition of the severity is mostly not clear and underlies the assessment of the surgeon. In a comparative review by Smeet *et al*., it was stated that, in all animal studies, bone graft or guided bone regeneration in addition to debridement had always better results than debridement alone [27]. To act as a successful augmentation material, each material needs to fulfil several properties. Key factors are a high biocompatibility, osteoinductivity and a certain mechanical stability [59].
A wide range of different materials were developed during the last decades. Bone grafts used in dentistry can be grouped in [56]:

- Autografts
- Allografts
- Xenografts
- Alloplast

Autografts derive from the same individuum. They have always shown good results and are seen as the gold standard in augmentation procedures [60, 61]. In dentistry, autografts are mostly harvested from the mandible [62]. But its major downsides are possible morbidity at the donor site, aesthetic outcome and restricted availability.
Allograft is bone material from the same species but different individual. In humans the material mostly derives from cadaveric human tissue. Allograft is rarely used in dentistry, since the availability is restricted and there is always the risk of the transmission of diseases.

A lot of effort was put in the development of bovine or porcine derived xenografts for bone augmentation. Xenografts show excellent characteristics regarding a high biocompatibility, osteoinductivity and a mechanical stability. Furthermore, they don't have the disadvantage of a restricted availability and the risk of transmission of diseases is lower. Thus, xenografts became a standard in peri-implantitis treatment.
Alloplastic bone augmentation materials are mostly biomaterials such as hydroxyapatite (HA) or tri-calcium phosphate (TCP). HA and TCP are known for their osteoconductive abilities. Their big advantages are their availability, possibility of sterilization and absence of immunoreactions [59]. Allografts can be administered in many different forms, like injectable paste, block or granules. And depending on their synthesis, not only the macrostructure, but the microstructure can be determined as well.
Nevertheless, in a study by Schwarz *et al*. (2008) a slight trend towards a better outcome when using natural bovine bone material (BioOss®) compared to nano-particles of hydroxyapatite (nHA) was seen in the treatment of peri-implantitis in human patients. It is also discussed, if xenografts might even have a better outcome than autografts [51].

1.4 Test item

The two test items, that will be investigated in this study consist of hyaluronic acid as a basis and hydroxyapatite as the augmentation material. In one test item, bisphosphonate is added to inhibit osteoclastic action.

1.4.1 Hyaluronic acid

Chemically it is assigned to the group of polysaccharide and virtually occurs in connective tissue of animals. It is known for its high biocompatibility. With its ability to release drugs, it is seen to be a very good carrier [63]. Furthermore, hyaluronic acid can be produced in a gel with a varying viscosity or a paste and therefore it can be used in various clinical situations [64]. Hyaluronic acid has become quite popular recently because anti-inflammatory and anti-bacterial capacities were shown. In addition, its well-known properties of tissue recovery make it a suitable candidate material in periodontal treatment. The ability of hyaluronic acid in dental surgery was proven by better healing of tooth sockets [65]. Martínez-Sanz *et al*. were able to show the ability of a hyaluronic based injectable hydrogel loaded with hydroxyapatite and bone-morphogenic protein-2 to promote bone growth in the jaw of rats [66].

1.4.2 Hydroxyapatite

Hydroxyapatite (HA) is known as a bone graft material for decades already. Reason is, that bone contains calcium phosphate in the form of nano-sized hydroxyapatite. This is the reason, why HA shows an excellent biocompatibility, biodegradability and no signs of toxicity [67, 68]. Hence, HA is potentially a perfect bone graft and since 1920, when the first successful implantation of a calcium phosphate (tri-calcium phosphate) was reported [69]. Since then, it was used in orthopedics and dentistry for various purposes such as implant coatings for enhanced osteointegration or bone graft materials [70, 71].

Different HA materials are claimed to have various *in vivo* abilities regarding osteoconduction and osteoinduction. It depends on the strut porosity as Chan *et al*. observed [72]. Reason is, that larger pores are easier to penetrate with bone structure, than smaller ones [73]. This is a reason, why researchers started to focus on HA again in 1995, when the fabrication of nano-sized HA was invented. The opinion was, that with a higher surface than in porous materials new possibilities in bone augmentation would be achieved [74]. And indeed, compared to conventional sized ceramics (grain size >100nm), a much higher proliferation of osteoblasts could be detected on nano-sized particles of HA in vitro [75]. Furthermore, it could be shown in vitro, that on cellular interaction, osteoclasts and osteoblasts react very similarly on nanoHA compared to natural bone. This is in contrast to ceramic HA [76]. Gotz *et al*. tested a gel based nano-crystalline hydroxyapatite in minipigs in subcutaneous and intramuscular tissue for biocompatibility and osteoinduction [77]. They observed formation of mineralised tissue within 5 weeks after implantation, which supports also osteoinductive properties of nanoHA in addition to the osteoconductive properties. Also, in the skull of minipigs, bone formation was shown in non-infected defects, which promotes the possibilities of peri-implant bone repair [78]. A fast mineralization of nHA in bone defects was seen within 12 was weeks.

Another feature of nHA scaffolds is their drug releasing ability. They have been loaded with antibiotics and resulted in a significantly better outcome due to continuous drug release [79].

Nano-crystalline HA is proven to help in reducing probing depth in patients suffering from peri-implantitis, but the long-term outcome might not be totally satisfying [80, 81]. The reason for this might be a poor remodeling rate, which was shown in different animal trials. Spies *et al*. saw a stagnation of bone repair after 6 weeks. The material itself is not

resorbed as shown in different studies [82, 83]. Campagnola *et al*. suggest the usage of nHA bone grafts with precautions, since their predictability is variable [84].

1.4.3 Bisphosphonates

Since many years, bisphosponates (BP) are especially known in the treatment of diseases with a high bone resorption, such as osteoporosis or Paget's disease [85, 86]. Over time, BP blazed a trail into oncology and now dental research also started to look for possibilities to treat peri-implant bone diseases with its help.

New insights in the mechanism of the two classes of BP could be achieved in recent years. It is known for several decades, that the molecules are able to inhibit the activity of osteoclasts. The molecules themselves have a strong affinity to hydroxyapatite [87]. Two classes of BP can be classified on their content of nitrogen and mechanism of action. One group does not contain nitrogen and has rather a low potential to inhibit osteoclasts by working via production of ATP metabolites that are toxic to osteoclasts [88]. The second group are nitrogen-containing BPs. Due to their strong binding abilities to HA, they will be taken up by osteoclasts during bone resorptive and remodeling processes. Within the osteoclast, they are able to inhibit farnesyl pyrophosphate synthetase [89]. It results in a destroyed cytoskeleton with cells undergoing apoptosis. On the other side, a possible inhibitory effect on osteoblasts is described, which would lead to less bone growth. But the mechanisms remain unclear [90, 91].

The effectiveness of BPs on implant stability has already been shown in animals two decades ago. Alendronate, a systemically administered bisphosphonate, showed good results in animal trials regarding a reduction of marginal bone loss in induced and naturally occurring periodontitis [92, 93]. Locally BPs can be administered as a coating of implants or directly at implant areas. Abtahi *et al*. could show that coated implants result in a better osseointegration and also a reduced marginal bone loss [94]. A coating with zoledronate, which is also part of the nitrogen-containing BPs, is also able to improve screw fixation in a rat model with screws inserted into the femur [95]. This is consistent with the findings in a study by Kettenberger *et al.* in which better implant stability through fast mineralisation was achieved using a gel with a combination of hyaluronic acid, HA and BPs than a control group without BPs. In addition, they could show higher bone formation rate with the help of zoledronate [96-98]. The delivery of BP's in form of a gel was tried before. In a randomized controlled clinical study local delivered low-dose alendronate could reduce probing significantly [99].

On the other side, it was shown that BPs coated HA implants have a much higher bacterial accumulation potential which then can lead to osteomyelitis. After BP's bind to HA, they still have free atoms. Bacteria take the free atoms as a point of fixation, which is the start for biofilm creation. A destabilisation of implants might happen and complete implant failure might be the long-term consequence [100, 101]. Although positive effects are undebated, one of the biggest problems seen in the treatment with bisphosphonates is their osteotoxicity in higher dosages. Bisphosphonate-related osteonecrosis in the jaw is seen in patients treated with systemic but also oral medication of bisphosphonate [102]. Furthermore, it was shown, that bisphosphonate have a cytotoxic effect on oral keratinocytes and fibroblasts which can potentially lead to wound healing deficiencies in the oral cavity [103, 104].

1.5 Animal model

Animal models are a critical step following *in-vitro* development and testing of a new material. Before entering a clinical phase, materials need to be safe for their use in humans and are therefore tested in animals. In addition, a lack of standardization, no possibility to generate histologic sections, risk of progression of diseases are just some of the disadvantages that human longitudinal studies face in dental research. In animals, materials can be tested for their effectiveness, biocompatibility, toxicity and side effects. There are different animal models preferred for different purposes.

Experimental dental studies in animals, are mostly carried out in cynomolgus monkeys and dogs. But in recent years, non-human primates and dogs are less and less used due to various reasons. Ethical considerations limit the use of these species. Dogs are rather seen as companion animals and nonhuman primates are rarely used because of their similarities to humans. Especially for nonhuman primates, regulations are very strict and costs for maintenance and housing are relatively high [105]. Studies in small rodents such as rabbits have been described, but due to limitation of intraoral space for implants and the difficulty to access to the oral cavity, this species is not a preferred model for dental implant testing. Furthermore, and more important, their tooth system differs considerably from human dental morphology such as lifelong growth of teeth, thus making them unsuitable for translation of results into human dental medicine.

Taking disadvantages into account, minipigs are a suitable alternative for various questions. Among the disadvantages, an aggressive and noisy behaviour in some animals and closer bond to the caretakers compared to rodents, should be mentioned. On the other

side, due to a similar tooth system, macrostructure and oral dimension, standard human implants can be used in minipigs as well. [106]. With an age of 24 months, minipigs have replaced their deciduous dentition with permanent teeth. While all deciduous teeth are similar in size compared to humans, permanent teeth are slightly larger [107]. Furthermore, regarding bone density, porcine bone is similar in trabecular and cortical bone to human bone because of its lamellar structure [108, 109]. The bone remodeling rate is around 1.0-1.5 µm per day in human. With a daily remodeling rate of 1.2-1.5 µm, pigs are quite close to human compared to dogs (1.5-2.0 µm/day) [110]. Also, the bone formation process is very similar to humans in defects filled with autologous bone. Within 4 weeks 39% newly formed bone could be detected in both species [48]. Furthermore, according to Hickey *et al*. minipigs show chewing similarities to humans. This results in similar loading mechanisms in both species. Overall, this makes minipigs a suitable model to investigate the safety and biocompatibility of biomaterials in dental research [106].

Next to the suitable animal that can answer a question, a model has to be chosen, that has the ability to answer one specific question at a time. Studies, evaluating the osteoinduction, osteoconduction or the biocompatibility, can be carried out in different areas of the body, such as trabecular bone, subcutaneous tissue or the hip bones [96, 111, 112]. The advantage of biologically and mechanically less complex areas is the possibility to investigate questions with less influencing variables that one has to consider in the interpretation. If the first step was successful, the question might become more complex at a second step.

In research on peri-implantitis ligature-induced peri-implantitis models are well established [113]. The model was first described by Lindhe *et al*. in 1978 in dogs [114]. One of the advantages in dogs is that the lesions achieved by ligature are similar to the naturally occurring ones in human [46]. Furthermore, once induced, the lesions rather show signs of progression, than spontaneous regeneration after ligature removal in dogs [115]. Later on, the model was translated to minipigs by Hickey *et al*. in 1991. In healthy animals, minipigs show an oral microbiota that consists predominantly of gram positive bacteria, even though the heterogeneity is bigger compared to human. The animals do not host peri-implantitis related microbiota in their oral flora, but they show the same pattern of a shift of gram positive in healthy to gram negative in animals suffering from peri-implantitis. The minipigs' inflammatory response is comparable to the one seen in

humans on both levels – macroscopically and microscopically. Macroscopically, they also show bleeding on probing, bone loss and pocket formation. In inflamed gingival tissue, miniature pigs also show an infiltration of inflammatory cells and vasodilatation on the microscopic level [116, 117]. Overall, the model is shown to be efficient, but it is still rare and to the author's knowledge, has only been described two more times [118, 119].

Another peri-implantitis model is a surgically created defect on the buccal wall. The model has different alternatives in shape: collateral defects, circumferential defects and excision of the buccal wall [120-122]. Through different shapes, the severity of the model can be influenced. With circumferential defects being the least and the excision being the most severe situation. Even though it does not reflect a naturally occurring peri-implantitis situation, it stands out because of its proclaimed standardisation and hence, its evidence on the efficacy of biomaterials.

1.6 Aim of the thesis

The goal of the study was to perform various pilot tests to evaluate an injectable biomaterial for peri-implant bone augmentation in minipigs. One injectable biomaterial consisted of hyaluronic acid and nano-particles of hydroxyapatite and another one had bisphosphonate in addition. The study was designed to provide preliminary data on the effectiveness and biocompatibility of the material as well as the suitability of the model.

2 Material and Methods

2.1 Study design

The study was divided in Part A and Part B. 8 miniature pigs were included in total, 4 dedicated to each part of the study. Two premolars and one molar were extracted of each mandible in each pig and were replaced by eight dental implants (4 in each hemimandible). In part A, acute bone defects at implant sites were created. In part B, ligature-induced chronic bone defects were provoked. In both parts, the bone defects were then augmented with test item 1 (TI1), test item 2 (TI2) or left empty (control).

Study Part	Group	Number of Animals	Number of defects treated with TI 1 or TI 2	Number of contralateral empty defects (Control)
A	TI 1	2	8 (4/animal)	8 (4/animal)
	TI 2	2	8 (4/animal)	8 (4/animal)
B	TI 1	2	8 (4/animal)	8 (4/animal)
	TI 2	2	8 (4/animal)	8 (4/animal)

Tab. 2.1: Study was divided in Part A and Part B and Test Item 1 (TI1) or Test Item 2 (TI2) was applied in each animal with control implants on the contralateral side. A total number of 8 implants were placed per animal

A split-mouth study design was chosen. The defects of one hemimandible were augmented with a test item (TI1 or TI2). The defects of the contralateral hemimandible acted as controls and were only flushed with 0,9% saline and left empty. Due to the pilot nature of the study no reference item was used (Fig. 7.2.1, 7.2.2). All implants were numbered with one being the most mesial and four being the most distal implant. The numbering was kept throughout the whole study (Fig.7.2.3).

Part A-animals were euthanised 6 weeks after test item implantation and Part B-animals were euthanised 4 weeks after test item application.

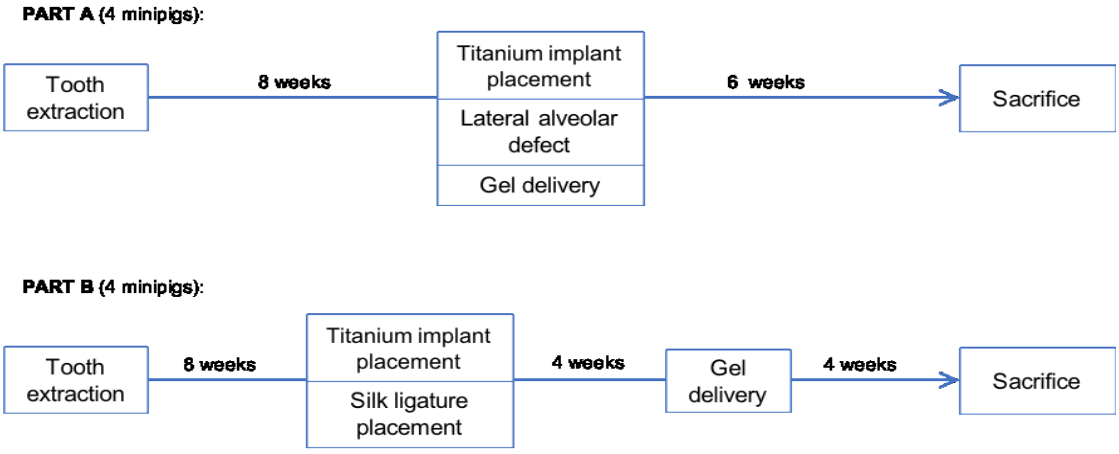

Fig. 2.1: Part A and Part B differed in the timeline and numbers of surgeries. Part A animals had to undergo two surgeries and Part B animals three surgeries.

2.1.1 Animal Testing

The study was authorised by the ethical commission of the Kanton Zürich under the permission number ZH263/16. The experiment was conducted according to the Swiss laws of animal protection and welfare (Tierschutzverordnung / Tierschutzgesetz).

2.1.2 Animals

The animals used in this study were Göttingen minipigs. All animals were female. Seven out of eight animals were former breeders (Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark). Upon arrival, their mean age was 33.75 months (26-38 months) and their mean bodyweight was 51.81 kg (47.7kg - 57.1 kg). The animals were marked with eartags and subcutaneous transponders (T-IS – Bio Glass Microchip, Datamars SA, Bedano, Switzerland). Clear identification was always possible throughout the study.

Housing was according to the Swiss requirements of animal protection and welfare (Tierschutzgesetz 455). Animals were housed in pairs or in a group of 4 as they had been housed in pairs at the breeder's facility. The group of 4 was monitored closely during acclimatization to ensure the animals exhibited a healthy social behaviour and were getting along with their box mates. Some animals had to be temporarily isolated after being attacked by its partner animals in the waking up phase after surgery.

The animals were acclimatized for 21 to 22 days under test conditions. They were clinically examined during the acclimatization period and checked at least twice a day for clinical signs during acclimatization period and during the in-life phase of the study. Only animals without signs of illness during the acclimatization period were included in this

study. During acclimatization period, animals were transitioned from solid to soft food. The food was moistened with approximately one-third solid food and two-thirds tap water in volume ratio. After each feeding, food for the next feeding was mixed in water to ensure sufficient softening. Animals were fed with a special minipig food (500g/day Mini-Pig Expanded, SDS Diet, Witham, Essex, England). Water was available *ad libitum*. The animals arrived from a microbiologically defined population (Ellegaard Göttingen Breeding Facility) and, before arrival, tested for various pathogens every six months. The last screening was two weeks before their transport to Zürich.
Animals were trained with a commercially available clicker as a noise stimulus and raisins as a reward. Animals could accustom to their new caretakers.

2.2 Biomaterial

Both, test item 1 (TI1) and test item 2 (TI2), were developed by the project partner. They consist of a biodegradable hydrogel paste containing components which are thought to enhance bone regeneration. Test item 1 was composed of a hyaluronic acid carrier with nano-particles of hydroxyapatite. Test item 2 had the same formulation as test item 1, with an added low dose of a bisphosphate. Due to pending patent processes, the exact composition remains confidential.

TI1	TI2
Hyaluronic acid	Hyaluronic acid
Hydroxyapatite	Hydroxyapatite
	Bisphosphonate

Tab. 2.2: Table shows the composition of the used test items.

The test items were delivered ready to use by the project partners, in individually wrapped sterilized applicators (Fig. 7.2.4). The test items were delivered and stored at ambient temperature in a dry, dark place and for longer storage (more than a week) were placed at a cool temperature (6-8°C).

2.3 Randomization

Animals were randomly selected and allocated to the study parts and the treatment groups during the acclimatisation period prior to first surgery.

2.4 Surgeries

2.4.1 Pre- and Perioperative management

Animals were fasted for at least 12 hours prior to surgery, with free access to water. The animals were sedated with azaperonum (1.51 – 4.00 mg/kg BW, i.m., Stresnil® ad us. Vet., Provet AG, 3421 Lyssach, Switzerland), atropine (0.01 – 0.2 mg/kg BW, i.m., Atropinsulfat Amino, Amino AG, Gebenstorf, Switzerland) and ketamine (18.87 – 40.00 mg/kg BW, i.m., Ketonarkon 100, Streuli Pharma AG, Uznach, Switzerland). The animals were examined for heart rate, respiratory rate, capillary refill time, mucosal colour, pulse strength or other visible signs of acute illness. After confirmation of the animal's health, the procedure was continued.

As soon as the minipigs were unconscious and examined, they were transported to the surgery room. An intravenous catheter (Introcan®, B. Braun Melsungen AG, Melsungen, Germany) was placed under aseptic conditions into the *Vena auricularis lateralis*. Blood samples were taken and sent to a laboratory for a blood analysis including hematology and blood chemistry.

From the implantation procedure on, pre and peri-operative analgesia was administered in all animals using buprenorphine (0.02 mg/kg BW, i.m. every 4 to 6 hours, Temgesic®, Reckitt Benckiser AG, Wallisellen, Schweiz) and carprofen (4 mg/kg BW, i.m. SID, Rimadyl® ad us. Vet., Zoetis Schweiz GmbH, Zürich). Prophylactic antibiotic treatment was installed on the day of surgery using amoxicillin and clavulanic acid (8,75 mg/kg BW, i.m., SID, Synulox®, Zoetis Schweiz GmbH).

As pigs tend to develop stress related gastric ulcer [123], a preventive treatment was administered in all animals using omeprazole (40 mg/animal, p.o., SID, Gastrozol®, Virbac, Hamilton, New Zealand) on the second and third surgery days.

2.4.2 Anesthesia

Anesthesia was induced with propofol (0.59 – 6.66 mg/kg BW, i.v., Propofol 1% MCT Fresenius, Fresenius Kabi (Schweiz) AG, Oberdorf, Switzerland) in the surgery preparation room. All animals were intubated intratracheally after local anesthesia of the larynx with lidocaine (Xylocain® Spray 10%. Aspen Pharma Schweiz GmbH, Baar, Switzerland). Anesthesia was maintained with vaporised isoflurane 2.0-2.5 Vol.-% (Attane™ Isoflurane ad us. Vet., Provet AG, Lyssach, Switzerland) in oxygen and air (Sulla 808V in combination with Vapor 19.3, Drägerwerk Schweiz AG, Liebefeld,

Switzerland). Oxyen/air ratio was 2/1. Additionally, intravenous propofol infusion (0.1ml/kg/h, i.v., Propofol 1%, Frsenius Kabi, Switzerland) was given. Constant rate was assured with Perfusor® (B. Braun Medical AG, Sempach, Switzerland). Animals were kept hemodynamically stable with Ringer-solution (5ml/kg/h Dr. G. Bichsel AG, Interlaken, Switzerland). A constant rate was assured using an electronic pump (Infusovet®, Eickemeyer, Tuttlingen, Germany).

The cardiovascular system was monitored during surgery (iPM10, Mindray Medical Germany GmbH, Darmstadt, Germany). Monitoring included electrocardiogram, heart rate and directly or indirectly measured systolic, mean and diastolic blood pressure. Direct measurements were performed through an arterial catheter (Surflo®, i.v catheter, Terumo Europe N.V., Leuven, Belgium) in the *A. auricularis lateralis* and indirect measurements with a cuff at the *A. coccygea ventralis*. Respiratory monitoring included respiratory rate and volume, inspired and expired concentrations of oxygen, carbon dioxide and isoflurane. During anesthesia, the cornea was protected with ophthalmic ointment (Vitamin A Augensalbe, Bausch & Lomb Swiss AG, 6301 Zug, Switzerland) and the animals were protected from hypothermia using a warmed air blanket (Bairhugger, 3M (Schweiz) GmbH, Rüschlikon, Switzerland).

Before each surgical intervention, both *Nn. mandibulares* were identified with a nerve stimulator (Stimuplex® HNS 12; B. Braun Medical AG, Sempach, Switzerland) and anesthetised with a perineural injection of ropivacaine (2 mL per nerve, Naropin®, 7.5mg/ml, Aspen Pharma GmbH, Baar, Switzerland).

2.4.3 Surgical Procedures

2.4.3.1 Tooth extraction (Part A & B)

Animals were placed in lateral or sternal recumbency and the mouth was kept open using a mouth gag and a cut syringe. Two premolar teeth and one molar tooth were extracted from each side of the mandible. First, an incision around the teeth was performed and a mucoperiosteal flap was elevated. Teeth with multiple roots were first divided with a diamond burr (iChiropro, Bien-Air Dental SA, Biel, Switzerland). Teeth were loosened and extracted using standard dental instruments such as forceps and elevators. Root remnants were removed using a root elevator and a drill (iChiropro, Bien-Air Dental SA, Biel, Switzerland). The extraction sockets were cleaned with 0.9% saline (0.9 % Natrium Cholratum, Grosse Apotheke Dr. G. Bichsel, Interlaken, Schweiz). Mucoperiosteal flaps

were repositioned and closed with single stiches using degradable suture material (Vicryl 2-0, Ethicon, Johnson&Johnson International, Diegem, Belgium))

2.4.3.2 Titanium implant placement, buccal defect creation and test item delivery (Part A)

Animals were placed in lateral or sternal recumbency and the mouth was kept open using a cut syringe. An incision was performed on the edentulous and healed crest and a full thickness flap was elevated. On each side of the mandible, 4 holes were drilled (iChiropro, Biel) under constant saline irrigation and a titanium implant was placed in each of them (SwishPlus® Implant, diameter: 4.1 mm, length: 10 mm, REF 924110, ImplantDirect, USA). The implants were placed in a 2 mm supracrestal position. Then, a bone defect on the buccal side (5 mm in width and 5 mm in depth) was created with a piezo surgical scalpel (Piezosurgery®, Mectron Deutschland Vertriebs GmbH, Köln, Germany).

Fig. 2.2: Sketch shows the defect creation of Part A (*Illustration Matthias Haab*)

The depth and the width of the defects were measured and noted. The defects were flushed with saline. Defect pockets were then filled with TI1 or TI2 on one side of the mandible using a syringe containing the test item and a 21G cannula. Defect pockets on the contralateral control side were flushed with 0,9% saline. Cover screws were inserted into the implants. A degradable, collagen dental membrane (Reguarde™ Resorbable Membrane, 30x40 mm, REF 16003040, Collagen Matrix, Inc, Oakland, USA) was cut to fit and two holes were punched into the membrane. It was then fixed with the cover screws to the most mesial and most distal implant. The membrane was then folded over the test item-filled defects side and the empty defects on the control side. No further fixation was

performed (Fig. 7.2.5). The mucoperiosteal flap was closed with single stiches using a degradable suture material (Vicryl 2-0, Ethicon, Johnson&Johnson International, Diegem, Belgium).

Fig. 2.3: Sketch shows the filled defects of Part A. The mucoperiosteal flap is retracted. Membrane is attached with the cover screws. ***(Illustration Matthias Haab)***

2.4.3.3 Titanium implant and silk ligature placement (Part B)

Animals were placed in lateral or sternal recumbency and the mouth was kept open using a cut syringe. An incision was performed on the edentulous and healed crest and a full thickness flap was elevated. On each side of the mandible 4 holes were drilled under constant saline irrigation and one titanium implant was placed in each hole (SwishPlus® Implant, diameter: 4.1 mm, length: 10 mm, REF 924110, ImplantDirect, USA). Abutments (RN Healing Cap, size: diameter: 5.5 mm, length: 4.5 mm, REF 048.037S, Institut Straumann AG, Basel) were placed instead of the cover screws. Cover screws were stored and re-sterilized. The mucoperiosteal flap was then closed and sutured around the implants with abutments with single stiches using a degradable suture material (Vicryl 2-0, Ethicon). Silk ligature material (Silkam® 2/0, REF 0762369, B.Braun Surgical, Rubí, Spain) was sutured around each abutment according to the method of Stübinger *et al*. [119] to provoke a development of peri-implantitis.

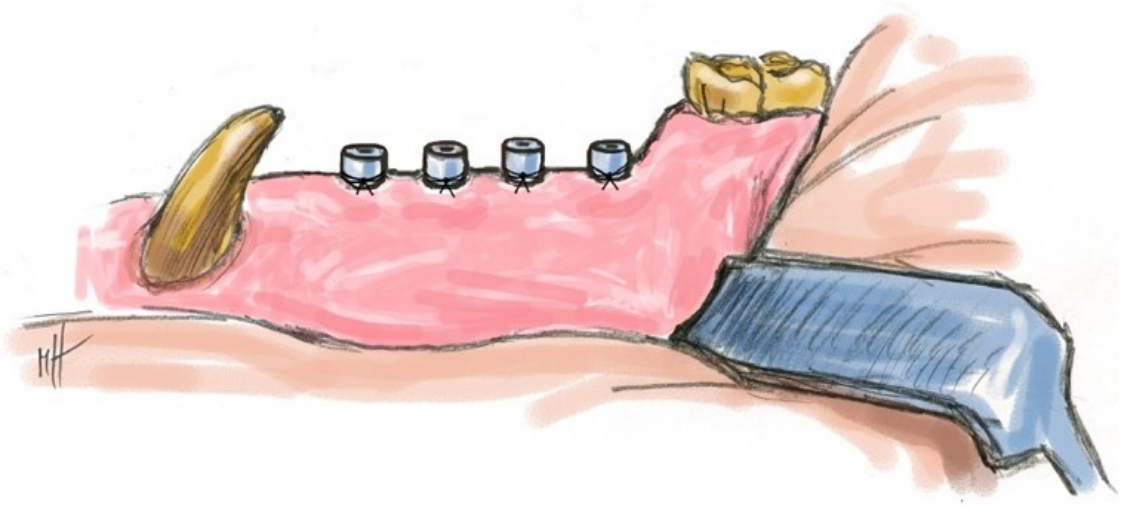

Fig. 2.4: Sketch shows the silk ligature placement around the abutments of Part B. The cheek is retracted. ***(Illustration Matthias Haab)***

2.4.3.4 Debridement, cleaning and test item delivery (Part B)

Four weeks after ligature placement, the animals in Part B were operated again. They were placed in lateral or sternal recumbency and the mouth was kept open using a cut syringe. All silk ligatures were removed. An incision was performed around the implants and a full thickness flap was elevated. The implant surface was mechanically cleaned using a rotating titanium brush (Straumann® TiBrush, REF 070.005, Institut Straumann AG, Basel, Switzerland). The defect areas were mechanically debrided with a gauze to remove all dead and inflamed tissue. Then, they were flushed with chlorhexidine (Chlorhexidine 0.1%, Dr. G. Bichsel AG, Interlaken, Switzerland). The depth of the defects was measured. The width was not measured, because the defects could not be differentiated from the neighbouring ones. The sterilised cover screws from 2.4.3.3. replaced the abutments. The defects were filled circumferentially with TI1 or TI2 with contralateral defects left empty (Fig. 7.2.6). A degradable, collagen dental membrane (Reguarde™ Resorbable Membrane, 30x40 mm, REF 16003040, Collagen Matrix, Inc, Oakland, USA) was cut to fit and two holes were punched into the membrane. It was then fixed with the cover screws to the most mesial and most distal implants. The membrane was then folded over the test item-filled defects side and the empty defects on the control side. No further fixation was performed. The mucoperiostal flap was closed with single stiches using a degradable suture material (Vicryl 2-0, Ethicon, Johnson&Johnson International, Diegem, Belgium).

Fig. 2.5: Sketch shows the filled defects of Part B. The mucoperiosteal flap is retracted. The membrane is attached with the cover screws. ***(Illustration Matthias Haab)***

2.4.4 Postoperatives Management

Antibiotic treatment was applied with amoxicillin with clavulanic acid perorally (1 pill/40 kg BW p.o., Clavubactin® 500 mg/125mg ad us. vet., Dr. E. Graeub AG, Bern) or, if animals were anorectic, as injection (8.75 mg/kg BW, i.m., Synulox®) for 4 to 6 days following the surgery day. As analgetic treatment, the pigs received caprofen as pills (1 pill/25 kg BW p.o., Rimadyl® 100mg ad us. Vet., Zoetis Schweiz GmbH, Zürich) and, if anorectic, as injection (4 mg/kg BW i.m., Rimadyl®) as long as were showing signs of reduced food uptake, but at least 4 days post-operatively. As a prevention from developing gastric ulcers, animals were treated with omeprazole (40 mg/animal/day p.o., Gastrogard®, Biokema, Crissier, Switzerland) as long as the animals were treated with analgesia. Furthermore, pain was also treated with buprenorphine (0.02 mg/kg i.m., Temgesic®, Reckitt Benckiser AG, Wallisellen, Switzerland) which could be administered every 4-6 hours, as long as deemed necessary, but mostly for one to two days.

2.5 In-life Observations

Medical records were kept for each animal. Animals were checked at least twice a day during there in life period for any clinical signs of illness: Alertness, posture, appetite, pain, respiration and lameness. All findings were documented in the medical record.
The animals were weighed once during acclimatization period on a scale for large animals (F-Star 125, Meier-Brakenberg, Extertal, Germany). Each time an animal was weighed,

the scale was calibrated using a 40 kg, 60 kg and 80 kg reference weight. Additional weighing was done once a month during the in-life period or within the week before the surgery.

Upon arrival, a physical examination was performed. An examination of cardiovascular and respiratory function was performed at day of anesthesia after sedation or if there were any signs of illness

2.6 Blood sampling

Blood samples were taken at each surgery day, 3 weeks after test item delivery and on sacrifice day. Serum (5 mL) and ethylenediaminetetraacetic acid (EDTA) (5 mL) samples were taken. At days of surgeries and sacrifices, blood samples were taken from the venous catheter. For the 3 weeks post Test Item implantation blood sampling, animals were fixated, but not sedated. Blood was taken from the *Vena jugularis* with a syringe and a canula.

2.7 Sacrifice and tissue harvest

Animals of Part A of the study were sacrificed 44 (+1) days after test item delivery. Animals of Part B were sacrificed 27 (+1) days after test item delivery. The animals were sedated as described in chapter 2.4.1 and an intravenous catheter was placed in the auricular vein. A blood sample was taken. Soft tissue around implant sites were probed using light stimulation with blunt forceps to check for bleeding.

Animals were then euthanised with pentobarbital (Esconarkon ad us. vet. 120 mg/kg BW, Streuli Pharma AG, Uznach, Switzerland).

Representative samples of kidney, liver and *Lymphonodi mandibulares* were taken. The mandibles were also freed from the surrounding tissue and harvested.

2.8 Radiographs

Post-mortem radiographs were taken of whole mandibles and after the mandibles were cut in half. The radiographs were performed using a faxitron machine (model: 43855A, Faxitron X-ray System, Hewlett Packard®, McMinnville, OR, USA). Radiographs were taken with 1 mAs and 60 V.

2.9 Micro-CT

Mandible halves were then stored in formalin 4% and scanned with a micro-CT (Skyscan 1076, Bruker micro-CT, Kontich, Belgium) at the Laboratory of Biomechanical

Orthopedics (EPFL, Lausanne, Switzerland). The scans were performed over 360° at full power of machine (voltage: 100 kV, current: 100 μA) with the use of a 1.0 mm aluminium filter and rotation step of 0.3° and an exposure time of 120 milliseconds.
After scanning, the mandible samples were returned to the Tierspital, Zürich for further embedding and histological processing.

2.10 Histological processing

The kidney, liver and *Lymphonodi mandibulares* samples were fixed in 4% formalin and stored, in case histopathological analyses would be needed in the future. They were not further processed as part of this study.
The mandible samples were dehydrated in an ascending ethanol series from 40-100%. After 7 days in Xylol, they were returned to 100% ethanol and ethanol series descended to 80%, to rise again to 100%. This alcohol series was chosen to prevent the samples of drying out completely when resting in pure Xylol for a longer period of time. This series gave space for possible delays.

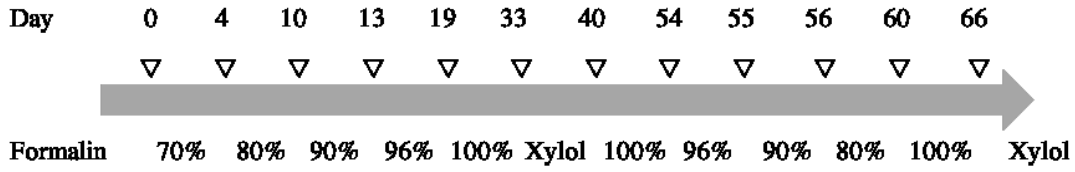

Fig. 2.6: Alcohol series; numbers in percentages is ethanol; triangles are indicating the timepoints of change of dilution

On day 60 of ethanol series, hemimandibles were cut with a diamond band saw (Exakt 300 CP, Exakt Norderstedt, Germany) to create 1 block per implant site. Samples were then placed in Xylol (Thommen-Furier AG, Rüti b. Büren, Switzerland)) for 14 days.
A mixture of 1 litre methylmethacrylate (MMA), 5 g perkadox 16 and 100 mL dibuthylphtalate (all 3 components: Sigma-Aldrich Chemie GmbH, Steinheim, Deutschland) was produced and homogenized with a magnetic mixer (Heidolph MR 3001 K, Laborbedarf, Schaffhausen, Switzerland) for 30 minutes. Samples were placed in glass jars which were filled with MMA mixture. After polymerizing, glass jars were crushed to free the sample blocks.
Only representative samples of Part A were further processed for sectioning (Tab.7.1**),** and all implants, but number one of Part B were processed for sectioning (Fig. 4.3).
Processing started with cutting the blocks along a marked line, parallel to the screw in lingo-buccal direction, using a band saw (Proxxon MBS 230/0, Oberrüti, Switzerland).

The surfaces of the blocks were smoothened with sand paper (grain size of 1200) on a rotating grinding machine (Struers Labor POL5, Struers GmbH, Birmensdorf, Switzerland). The blocks were then dried for at least 8 hours at 37 °C. After drying the blocks were glued on transparent PMMA slides (PMMA GS transparent, 75x25x3 mm, Maagtechnic AG, Dübendorf, Switzerland) with Technovit® 7210 VLC (Morphisto GmbH, Frankfurt, Germany). The blocks were glued as parallel as possible to the slide. If the block was not parallel, wax (Modellierwachs 1.25 mm, Belladi Ruscher Schleusser GmbH, Amriswil, Switzerland) was glued between block and slide on one side. Technovit® was polymerised with blue light for at least 15 minutes (Exakt 402, Exakt, Norderstedt, Germany). The block was then cut longitudinally through the implant axis and parallel to the slide with a diamond band saw (EXAKT 300 CP, EXAKT, Norderstedt, Germany). Samples were cooled with tap water during the cutting processes. For exact parallel cuts, slides were attached to the saw with a vacuum pump. Then, samples were ground with sand paper (grain size of 1200) on a rotation grinding machine (Exakt 400CS, Exakt, Norderstedt, Germany) to make sure the surface of the block was parallel. Grinding was also done under cooling using tap water. The thickness of the block plus the slide was measured with a measuring device (IP65, Mitutoyo (Schweiz) AG, Urdorf, Switzerland). It was then dried again, and a second slide was glued on top of the block using a vacuum system. Before gluing, the thickness of the second slide was noted. After gluing, the thickness of the whole sandwich slide was noted, and the thickness of the glue layer was calculated. With a diamond band saw, a slice was created to a thickness of approximately 200 µm on the second slide. It was then grinded down to 150 µm on the same grinding machine used before as well (grain size of 800) and further to 70-80 µm with a grain size of 1200. The slide was then polished using a grain size of 4000 for 10 minutes. In the end, slides were polished with diamond pastes (3 µm and 1 µm, both DP-Suspension P, Struers GmbH, Birmensdorf, Switzerland) for one minute each. A thickness of 70-80 µm was pursued.

The slides were then stained with Sanderson's Rapid Bone Stain (Dorn & Hart Microedge Inc, Loxley, USA) and counterstained with acid fuchsin (Dorn & Hart Microedge Inc, Loxley, USA), dilution 1 to 4 in water. The colour distribution of this stain is:

- Soft tissue and osteoid: blue
- Mineralised bone: pink

Representative samples were also surface stained with toluidine blue.

2.11 Analyses

2.11.1 Blood sample analysis

The blood was analysed for inflammatory and toxicity responses via routine hematologic analyses and clinical chemistry. The blood samples were analysed at the laboratories of the Tierspital (Veterinary Clinical Laboratory, Vetsuisse Fakultät, University Zurich, Switzerland).

2.11.2 Macroscopic evaluation

A semi-quantitative evaluation of macroscopic assessment was done post mortem, focusing on the area containing implants and there on inflammatory reactions, signs of poor biocompatibility, bone formation and repair and on stability of the implants themselves. The evaluation was done by a senior scientist in a blinded manner. The macroscopic evaluation included the assessment of redness, swelling, mucosal surface, inflammation, breach of implants through the mucosa, and soft tissue hyperplasia. Implants were examined for possible loss of cover screws, possible loosening and the space between the implants (interspace). The smaller interspace between two implants was taken into account for the scoring. All findings were translated into individual scores and finally a total score was calculated.

ROI	Parameter	Score							
Gingiva	Redness	0	none	1	mild	2	moderate	3	red
	Swelling	0	no	1	mild	2	moderate		
	Surface	0	smooth	1	cobblestone	2	eroded		
	Inflammation	0	none	1	mild	2	moderate	3	severe
	Breach	0	covered	1	half covered	2	transgingival	3	threads visible
	Hyperplasia	0	none	1	mild	2	moderate		
Implant	Cover screw	0	on	1	half on	2	off		
	Firmness	0	firm			2	loose		
	Interspace	0	> 4mm	1	2-4mm	2	<2mm		

Tab. 2.3: Scoring system for macroscopic evaluation

2.11.3 Radiographic analysis

Radiographs were assessed for a radiolucent zone around implants and bone resorption of surrounding bone (between implants). Radiographs were evaluated by a senior scientist in a blinded manner. A subjective score was given for each implant by the senior scientist. Bone resorption in the interspace was calculated for each implant by taking the average of the two neighbouring scores. For implant 1 and 4 only the mesial (implant 1) or distal

(implant 4) was taken into account. Since its limited standardisation and no base line, radiographic results were not included in an overall score.

Radiolucent zone around implant	Bone resorption interspace	Sclerotic rim around implant
0 no halo (0% of implant)	0 none	0 none
1 partially (1-25% of implant)	1 mild	1 mild
2 moderate (25-50% of implant)	2 moderate	2 moderate
3 severe (>50% of implant)	3 severe	3 severe

Tab. 2.4: Scoring system for radiologic evaluation

2.11.4 Micro-CT analysis

Micro-CT analyses were performed at the Laboratory of Biomechanical Orthopedics (EPFL, Lausanne, Switzerland) to account for bone mineral density changes and microscopic changes in bone structure.

2.11.5 Histological evaluation

Sections were photographed with a macroscope in combination with a digital camera (Leica Z6 APO A, Leica DFD 450 Digital Camera, Leica Macroscop Smart Touch control unit, Leica Microsystems AG, Heerbrugg, Switzerland; ImageAccess 12 Standard, Imagic Bildverarbeitung AG, Glattbrugg, Switzerland).

Only RBS stained sections were evaluated. Sections were observed using a microscope (Leica DMR Systems, Leica Microsystems AG, Heerbrugg, Switzerland) and semi-quantitatively scored. Only the region close to the implant was evaluated (Fig. 7.2.7). The inflammation grade, type and location as well as presence of foreign body giant cells and remnants of collagen membrane were noted. The tissue sections were explored for remnants of the test item. In the region close to the implants, the bone was graded for bone activity, accounting for both, resorption and formation. This was done for the buccal and the lingual side separately. In addition, the presence of osteoclasts and presence of necrosis and osteolysis was noted. Histological samples were evaluated by a senior scientist in a blinded manner. Results were then translated into a score.

Inflammation grade	Bone activity
0 none	0 formation
1 mild	1 static
2 moderate	2 resorption
3 severe	

Tab 2.1: Scoring system for histological evaluation

Furthermore, the region far from the implant was examined and notes were given on the overview.

Pocket depth, pocket width, bone resorption, epithelial invasion and soft tissue thickness were measured and noted in microns (Fig. 7.2.8).

2.11.6 Data analysis and statistics

Descriptive statistics including plots (scatter plots and box plots) were done using SPSS 23.00 (IBM Corp., Armonk, NY, USA). No statistical comparisons were done, seeing that the study was only a pilot and number of animals and samples was small.

3 Results

3.1 Animals

None of the animals had to be euthanised prematurely. Results of all animals were included in the study.

Animals showed signs of anorexia during the project for some days. Anorectic periods occurred especially on surgery days post-operatively, but also during periods unrelated to surgeries. All animals were treated with a proton pump inhibitor, omeprazole (Gastrogard, p.o, Biokema SA, Crissier, Switzerland). Two animals were anorectic for more than 7 days (85.05, 85.08). All other animals did not show prolonged anorexia. Additional in-life radiographs of the mandibles (85.05) were taken to rule out study related anorexia. While remnants of the premolar roots were seen on the pictures, this was ruled out as the reason for anorexia, because animal started eating shortly after again (Fig. 7.2.9).

During anorectic periods, animals 85.05 and 85.08 had high levels of eimeria and *brachyspira pilosicoli* detected (both were detected in the faeces). All animals were then treated preventively. Eimeria were treated with Toltrazuril (0.4 ml/kg, p.o., Baycox® 5% Provet AG, Lyssach b. Burgdorf, Switzerland) and brachyspira pilosicoli were treated with Tiamulin (4 mg/Tier, p.o., Denagard® 10%, Elanco, Basel, Switzerland).

Animal 85.05 developed an abscess at the lateral abdomen after fighting with another animal. This could have resulted in alterations in blood results. The abscess was split during second surgery and healed without complications.

3.2 Surgery

3.2.1 Tooth extraction (Part A+B)

All surgeries were carried out as planned. There were no major complications during extraction process. Parts of tooth roots were left inside the mandible because it was not possible to clear the sockets completely without destroying the alveolar bone socket. Remnants of tooth roots were first seen in in-life x-rays of 85.05 (Fig. 7.2.9). It was decided to leave the remnants in the mandible. Postmortem radiographs (Fig. 7.2.18, Fig. 7.2.19) and micro-CT confirmed presence of multiple roots. However, all animals recovered well after surgery. 85.07 showed signs of apathy the next day after surgery, but it resolved shortly after.

3.2.2 Titanium implant placement, buccal defect creation and test item delivery (Part A)

The implantation process could be carried out as planned and without any major complications for Part A. Some implants were placed with a slight deviation from vertical direction and also from a centred line on the alveolar crest (e.g. 85.02 L2).

All of the created defects had similar sizes. Defect sizes were around 5 mm in depth (mean: 5.17 ± 0.53 mm) and 5 mm in width (mean: 4.86 ± 0.53 mm). The defect of one implant was larger in depth (7mm in 85.01 R1, Ctr) and in another implant, width was 2 mm smaller than the aimed 5 mm (3 mm in 85.01, R3). In depth, defect sizes were slightly bigger on the right side (5.28 ± 0.71 mm) compared to left sided implants (5.06 ± 0.25 mm) (Tab. 7.1.2).

In general, cutting lines were not as clear in 85.01 R than in the rest of the animals. TI was applied as planned. One syringe of 3 ml was used per animal. In one animal (85.01) the membrane was not fixed with cover screws on the control side because it was large enough to hold itself onsite.

According to the surgeon, the mucosa was closed on top of the implants under tension. All animals recovered well after surgery.

3.2.3 Titanium implant and silk ligature placement (Part B)

Implantation process could be carried out as planned for Part B. Some implants were placed with a slight deviation from vertical direction and also from a centred line on the alveolar crest (e.g. 85.08 L3). An extra hole had to be drilled for implant 85.07 R1. Reason was a lack of buccal wall stability. Soft tissue was closed around the implants with abutments without tension. Silk ligature was applied as planned. All animals recovered well after surgery.

3.2.4 Debridement, cleaning and test item delivery (Part B)

Debridement, cleaning and test item delivery was carried out as planned. All abutments and head of implants presented themselves with a covering biofilm. Within the biofilm, remains of food were seen (Fig. 7.2.10). After the mucoperiosteal flap was elevated, clear view on implants with covering granulation tissue and plaque was possible. The surrounding tissue was hyperaemic. No pus was detected in any animal.

Abutment and silk ligature of 85.06 L4 were lost prior to surgery. The timepoint of the abutment loss remains unknown. One animal (85.07) showed excessive bleeding during

debridement of the right sided implants. This site presented itself covered with more granulation tissue compared to other implants.
The defects were only measured in depth. The reason was, that crater shaped defects were confluent (Tab. 7.1.2). Mean depth in Part B animals was 3 mm.
According to the surgeon, closing the flap on top of the implants was very tight for all the animals of Part B. It was not possible to attach the mucosa on top of the implants on the left side of 85.08. Therefore, mobilised sublingual tissue was rotated on top of the implants. Due to the flap it was possible to close the mucosa on top of the implants without moderate tension. Sublingual tissue was reattached with resorbable suture material Vicryl 4-0 with single stiches (Fig. 7.2.11). All animals recovered well after surgery

3.3 Sacrifice

Sacrifice was done six weeks after test implantation in Part A and four weeks after TI implantation in Part B. All animals were sacrificed as planned.
In Part A and Part B animals, the bleeding on probing was positive after mild manipulation.

3.4 Analyses

3.4.1 Blood results

Results of blood samples differed between timepoints and animals. But no relation to Test Item placement could be drawn.

3.4.2 Macroscopic evaluation

3.4.2.1 Part A

In Part A, all 32 implants were included in the macroscopic evaluation. Evaluation was carried out according to the protocol. Straw, hey and remains of food were found around the exposed implants and had to be removed for evaluation. All implants of Part A presented themselves not or only half covered with mucosa (Fig. 7.2.12). The surrounding mucosa was moderately to severely reddened. No pus was detected. Mucosa was not swollen around any implant, the surface presented itself cobblestoned to eroded. It was undergoing a mild to severe inflammation. The severity of the inflammation differed between animals but did not within the same animal. Differences could be seen in the

location of the inflammation: the mucosa of the buccal side presented itself worse regarding erosive inflammation and redness compared to the lingual side. Threads of most implants were visible on the buccal side. In some cases, though, the mucosa had built bridges in the interspace of the implants (85.03 L1/2).

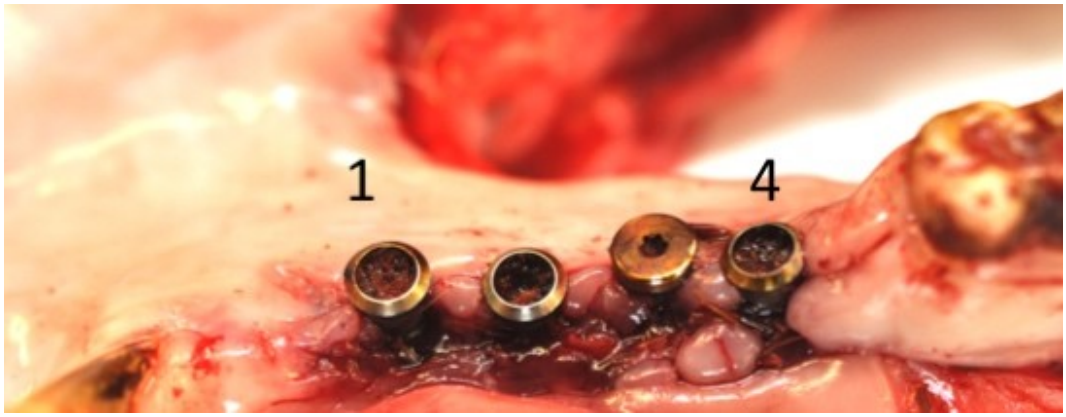

Fig. 3.1: On this picture of the left hemimandible of animal 85.02, no implant is covered with mucosa. The mucosa around the implants is reddened compared to the surrounding tissue. Cover screws of implants 1,2 and 4 are lost.

When manipulating the implants, it was possible to move eight of twelve implants (Ctr 5/16, TI1 1/8, TI2 2/16). 85.04 L1, was sticking out almost 50%. Cover screws were lost or loosened in TI animals and Ctr animals (Fig 3.1). More cover screws were loose or lost in position 1 and 4 (9/11 cover screws that are lost or loose). Only two cover screws were lost or loose in positions 2 and 3. In 6 implants, the interspace was more than 4 mm. In the rest of the implants, the interspace was less. In one implant hypertrophic soft tissue was detected (85.01 L4). No suture material could be detected. No remnants of the membrane were visible.

As a first impression there could be no difference seen between test item groups and control groups (Tab 7.1.3). In redness TI2 and Ctr did slightly better (Ctr: 1.7 ± 0.45; TI2: 1.7 ± 0.46) compared to TI1 (2.88 ± 0.35). The inflammation was least in TI2 (1.75 ± 0.46) compared to TI1 (2.50 ± 0.53) and Ctr (2.69 ± 0.48). TI1 group had the best results in firmness (0.25 ± 0.71), followed by TI2 (0.50 ± 0.93) and Ctr (0.63 ± 0.96). In the total scoring, TI2 performed slightly better (10.25 ± 2.05) than Ctr (11.19 ± 2.56) and TI1(12.50 ± 3.02) (Fig. 3.2). More detailed results can be found in Fig. 7.2.13 and Fig 7.2.14.

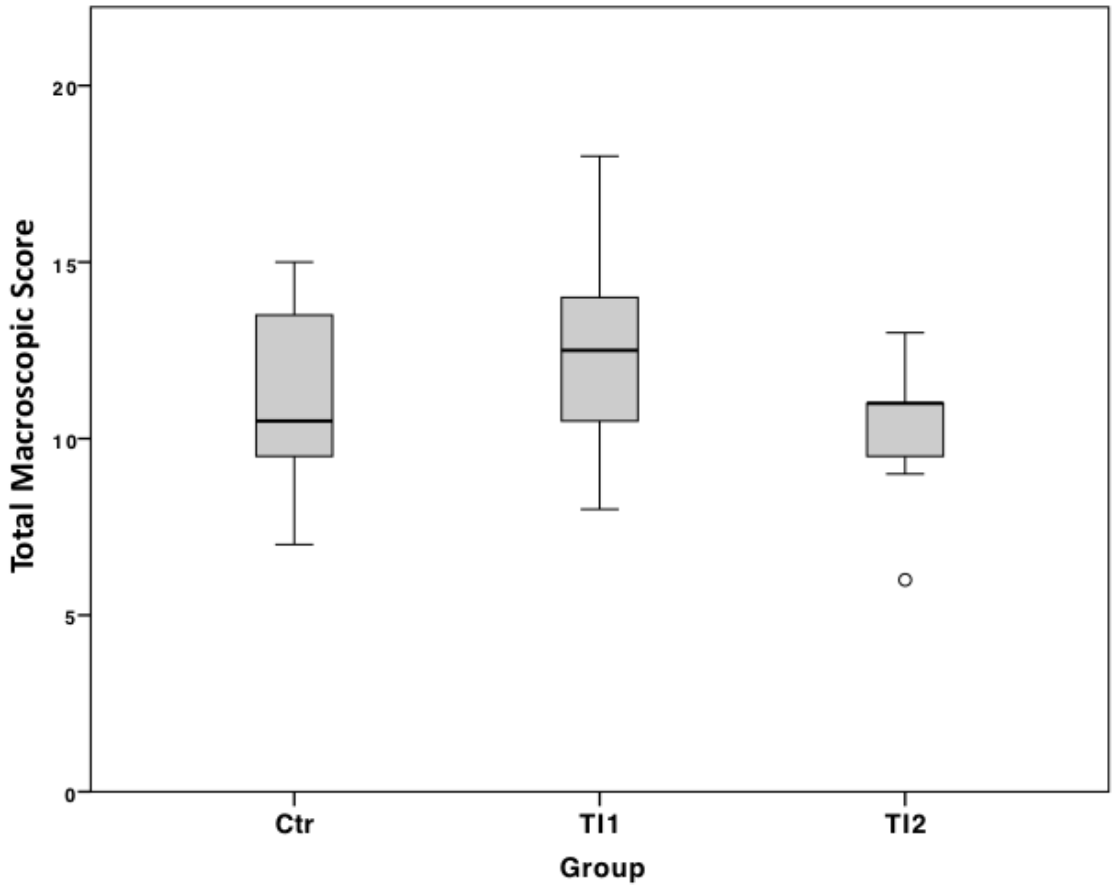

Fig. 3.2: The plot shows the Total Macroscopic Score (0-21; 0 being the best score) in relation to the treatment groups
o: 85.03 L1

3.4.2.2 Part B

In Part B, all 32 implants were included in the macroscopic evaluation. Macroscopic evaluation was carried out according to the protocol. Straw, hey and remains of food were found around the exposed implants and had to be removed for evaluation. All implants of Part B presented themselves not or only half covered with mucosa (Fig. 7.2.15). Compared to Part A, mucosa presented itself less reddened (Fig. 3.3). The inflammation was still mild to severe depending on the animal. In one mandible (85.08 L) the mucosa was swollen, in the other animals, no swelling was detected. In all animals, the surface of the mucosa was smooth to cobblestoned. In Part B, both mandibles of each animal had the same peri-implant surface. The mucosa was inflamed in all animals (mild to moderate). No pus could be detected in any animal. In Part B animals, none of the sides was completely covered with mucosa. Mucosa had built bridges in the implant interspaces. All implants were firm. One cover screw was lost (85.08 L1). Remnants of suture material was found in all mandibles. In some animals a hypertrophy of the mucosa

was detected (7/32 implants). No remnants of the membrane were visible. All implants were placed more than 4 mm away from each other (except 85.08 L1).

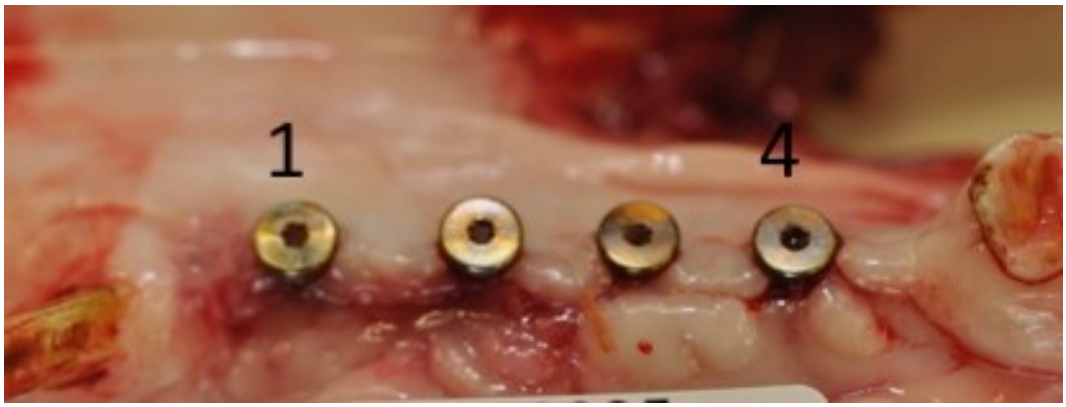

Fig. 3.3: The left hemimandible of 85.05 is visible. Implants number 1 and 4 are indicated. All implants are exposed. The mucosa around the implants is slightly reddened. All cover screws are on site.

At first impression, there were no differences between TI groups and control group (Tab. 7.1.4). However, control implants were less reddened (1.75 ± 0.77) compared to TI2 (1.88 ± 0.83) and TI1 (2.00 ± 0.93). Mucosal surface was the calmest in TI2 (0.50 ± 0.53) compared to Ctr (0.81 ± 0.40) and TI1 (1.0 ± 0.00). Inflammation was the least in TI2 (1.50 ± 0.53) followed by TI1 (1.63 ± 0.52) and Ctr (1.69 ± 0.60). In the total score, TI2 performed the best (6.00 ± 1.77) followed by TI1 (6.75 ± 1.58) and Ctr (7.13 ± 1.67) (Fig. 3.4). More detailed results can be found in Fig. 7.2.16 and Fig 7.2.17.

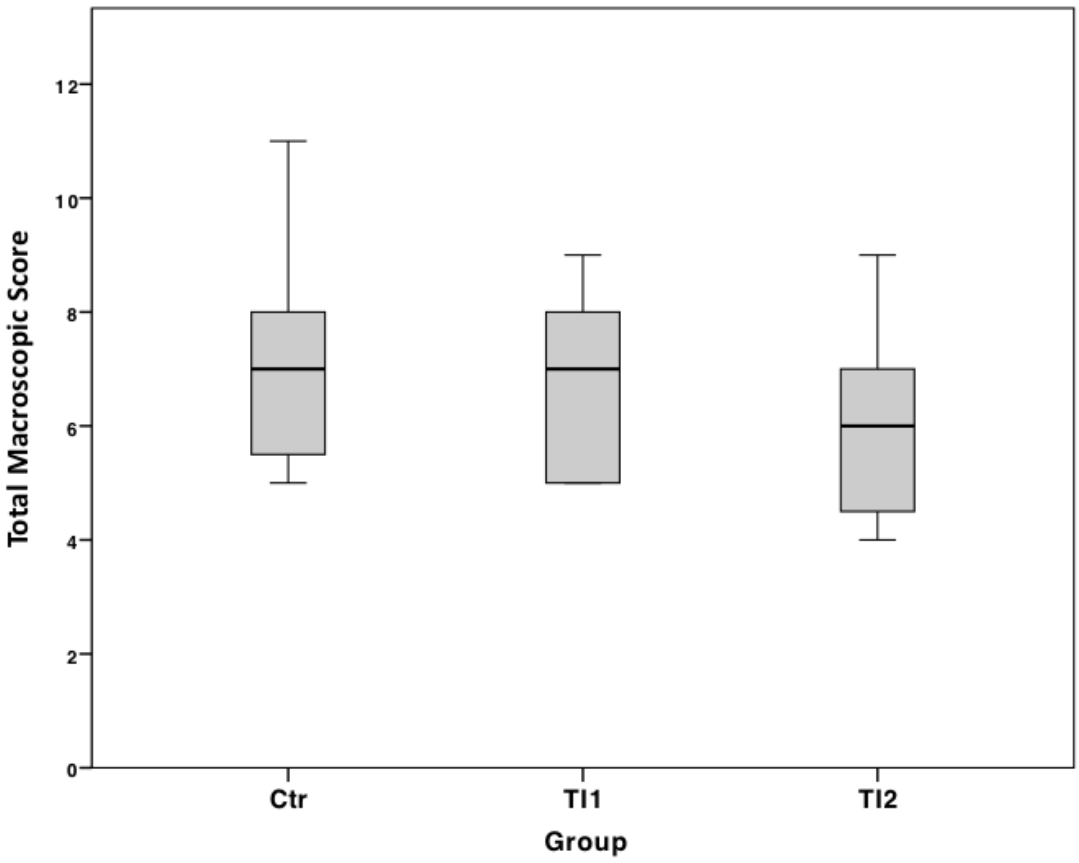

Fig. 3.4: Boxplot showing the Total Macroscopic Score (0-21, 0 being the best score) in relation to the treatment group

3.4.3 Radiologic images

3.4.3.1 Part A

Radiographs were all evaluated. Radiographs revealed that all number one positioned implants, but 85.03, were placed into the root of the canine (Fig. 7.2.18). These implants (85.03 as well) were excluded from the evaluation of the radiolucent zone. No radiopaque areas were detected on the radiographs, that could resemble the test item. The Ctr sites (1.63 ± 1.02) showed the least bone resorption in the interspace of the implants compared to TI1 (1.69 ± 0.80) and TI2 (2.31 ± 0.75). Looking at the implants themselves, TI2 (2.33 ± 0.82) had a smaller radiolucent zone than Ctr (2.50 ± 0.52) and Ti1 (2.50 ± 0.55). The bone density of 85.03 was reduced compared to the rest of the animals (Tab. 7.1.5).

3.4.3.2 Part B

Radiographs were all evaluated. Radiographs revealed that all number one positioned implants were placed into the root of the canine (Fig 7.2.19). These implants were

excluded from the evaluation of the radiolucent zone. No radiopaque areas were detected on the radiographs, that could resemble the test item. Similar to Part A, Ctr (0.09 ± 0.27) sites showed less bone resorption in the interspace compared to TI2 (0.19 ± 0.37) and TI1 (0.50 ± 0.80). Implants of TI2 (0.67 ± 0.82) showed a smaller radiolucent zone if compared to Ctr (0.75 ± 0.87) and TI1 (1.50 ± 0.55) (Tab. 7.1.5).

3.4.4 CT

Radiographic observations were confirmed by micro-CT. In Part A and Part B, all implants number one (except 85.03) were placed in the root of the canine. Implants of 85.02 L2 and 85.07 L2 were placed in the root of the canine as well. In minipig 85.03 left side, a remaining tooth was found under the bone surface, implant number two and three were placed into this tooth.

In general, it was found that the bone reaction to the treatment was violent in Part A and comparatively moderate in Part B. In Part A, 8/32 implants were loose (Ctr 5/16, TI1 1/8, TI2 2/8). The bone surface in Part A was rough. No loose implants were found in Part B. As the test item materials mineralize, it might be possible that some of those structures (especially when they are not linked to the bone) might be mineralized gel. This possibility could not be excluded, since CT does not distinguish calcium between mineralized bone or hydroxyapatite.

3.4.5 Histological evaluation

In Part A only representative samples were processed for histology. They were only screened for possible remnants of test item, membrane or mineralized tissue Part A was not evaluated. In Part B all samples except number one positioned implants were processed and evaluated according to the protocol.

3.4.5.1 Part A

The severe bone loss and inflammation, as already seen macroscopically, were microscopically confirmed. No remnants of the test item could be detected. Accordingly, no remnants of the collagen membrane were observed. Mineralized tissue was not observed.

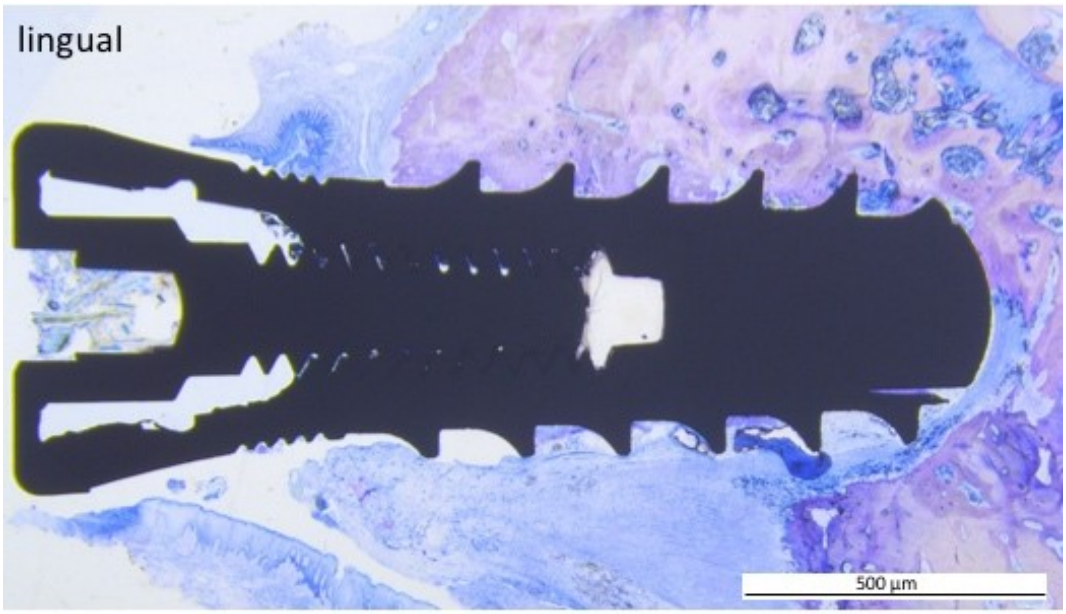

Fig. 3.5: 85.02 L2: purple is mineralized bone and blue is soft tissue. Defects were created on the buccal side. In this picture, there is no evidence of defect healing on the buccal side. Magnification: 7.11 x 1.0; Sanderson's RBS

3.4.5.2 Part B

In Part B, all samples but position 1, could be stained and evaluated and presented themselves very similar (compare Fig. 3.6).

The histological samples showed an inflammation grade from "no inflammation at all to a severe inflammation" in one sample (85.06 L2). Evaluation of the slides was hindered by black particles that probably derived from implant particles during the grinding process (Fig. 7.2.20).

If there was an inflammation, it was mostly of mononuclear nature. Predominantly plasma cells and lymphocytes were observed. In 85.07 L2 though, the inflammation was more of polymorphonuclear nature. Here, mainly neutrophilic leucocytes were observed. Single cells were spread around within the soft tissue. No foci could be determined. Foreign body giant cells were not observed., but in 85.04 R4 and 85.06 L2 around collagen tissue and in 85.06 L3 around a big structure, that could be part of the suture (Fig. 7.2.21). In this sample it was spread around possible remnants of the collagen membrane. Microscopically, unstructured collagen fibres were observed within the soft tissue in 12 out of 24 samples. They were mostly lined at the bone/soft tissue boarder. These collagen fibres were possible remnants of the collagen membrane. They could be remnants of dissolved bone as well. No test item (gel) was found. No mineralized tissue was found.

On the buccal side, bone activity was mostly formation on w, whereas on the lingual side predominantly resorptive processes were going on were noticed (Fig. 7.2.22).

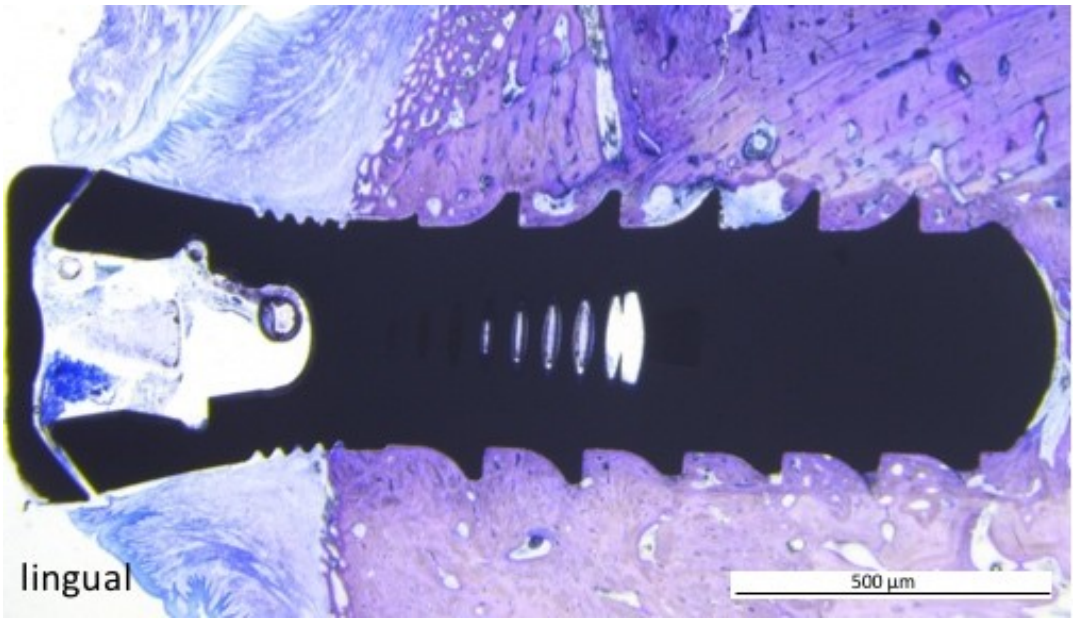

Fig. 3.6: 85.07 L4: purple is mineralized bone and blue is soft tissue. Circumferential defects developed through ligature placement. No evidence of new bone formation is visible on this picture.
Magnification: 7.11 x 1.0; Sanderson's RBS

Some implants were not well integrated within the bone. Loss of bone contact occurred at regions all around the implants. However, there was no implant with a complete loss of bone/implant connection.

Histological measurements were sometimes hindered by the search for the highest point when soft tissue was densely folded like 85.06 L2. In these cases, the folded part was counted into the soft tissue thickness. There was one outlier with extraordinary high pocket depths (85.07 L2). The same implant was placed into a root of another tooth. Even though having no bone contact on the buccal side, it showed reasonable good osseointegration on the lingual side. All results are summarised in Table 7.1.6. On the buccal side, more bone formation was seen in Ctr and TI2 (Ctr: 0.50 ± 0.80, TI2: 0.50 ± 0.84), but in TI1 processes were more resorptive (1.33 ± 0.52). Within the resorptive processes on the lingual side, TI2 showed less resorption (1.17 ± 0.98) compared to Ctr (1.33 ± 0.52) and TI1 (1.50 ± 0.80). For the total score, TI2 (2.83 ± 2.14) performed better compared to TI1 and Ctr (Ctr: 3.33 ± 1.67, TI1: 3.33 ± 1.21) (Fig. 3.7, Fig. 7.2.23).

Within the TI2 group of Part B only 2 had pockets (2/12), whereas 3/12 in TI1 group developed pockets and 8/12 of the Ctr group. Measurements were done twice: Including the depth of 85.07 L2 and excluding the sample. Including the depth, TI1 (25.83 ± 29.57 µm) and Ctr (42.33 ± 50.65 µm) performed better than TI2 (103.50 ± 29.07 µm) (Fig.

7.24, Fig. 7.25). But mean pocket depth drops down to 13.00 ± 29.07 µm when excluding 85.07 L2. On the buccal side, soft tissue was TI2 (355.33 ± 124.24 µm) thicker compared to TI1 (340.67 ± 98.87 µm) and Ctr (349.42 ± 100.62 µm) (Fig. 7.26). Hence, epithelium invaded the least in TI2 into the soft tissue/bone cavity (Ctr: 272.50 ± 103.9 3µm; TI1: 290.50 ± 60.89 µm, TI2: 261.60 ± 117.16 µm).

4 Discussion

4.1 Minipig as a peri-implantitis model

One of the first things, when considering an animal study, is the choice of a suitable model. This decision is crucial for the success of the study. For peri-implantitis studies, different animal models are described in nonhuman primates, dogs, rodents and minipigs. Most studies are carried out in nonhuman primates and canines, though. Minipigs on the other side, are popular for the evaluation of biocompatibility and efficacy of new materials. Studies investigating peri-implantitis and its treatment possibilities are described in minipigs only rarely [106]. This seems to have several reasons, but the animals' restrictive compliance to manipulation in the oral cavity is one of them. In minipigs, it takes some effort to train the acceptance of manipulation in the oral cavity without being stressed [124]. This acceptance is one of the key factors for the success of a peri-implantitis study. The reason is, that post-surgical oral hygiene is not only important as a follow-up treatment limiting the influencing effects of food, straw and bacteria [125]. Furthermore, during oral hygiene, insights in the oral cavity are possible and surgery wounds can be monitored.

On the other hand, peri-implantitis studies are carried out in minipigs as well, because of their well-known reaction patterns regarding the biocompatibility and efficacy of new materials. In addition, the lower costs in maintenance make minipigs an interesting alternative. In contrast to dogs and non-human primates, minipigs are easier to justify ethically. In western societies, dogs are more seen as companion animals and non-human primates are too close to humans.

In the last decades of research on peri-implantitis, most of the studies were carried out in healthy animals. But there is a strong need to investigate the patterns of peri-implantitis in compromised animals as well. Minipigs may serve as an animal model for experiments in animals suffering from diabetes mellitus [106]. It was successfully shown, that a diabetes model for the investigation of the osseointegration of dental implants in minipigs is possible [126]. Hence, there might be more studies carried out in miniature pigs in the future. However, the animal model used in the current study had severable problems that need to be addressed before minipigs would further serve for preclinical studies in dentistry.

One challenge encountered in the present study is the sensitivity to pain related stress in miniature pigs. When stressed, pigs can develop gastritis, progressing to gastric ulcer if stress is not reduced [124]. In addition, nonsteroidal anti-inflammatory drugs, traditionally given post-operatively, like carprofen raise the susceptibility of developing gastric ulcer. Clinically, affected animals commonly show signs of anorexia among other nonspecific signs. The problem is that without further examinations, it is impossible to differentiate between gastritis-related or study- and pain-related anorexia. Therefore, from the implantation process on, animals were preventively treated with omeprazole. Omeprazole is a proton pump blocker, which reduces the risk of gastric ulcer by raising gastric pH and thereby protects the gastric mucosa.

One other reason for anorexia we faced were infections with *brachyspira pilosicoli* and eimeria. After targeted treatment, animals started eating again and study related anorexia was less likely. If animals are raised in a specific-pathogen free environment, such as those in this study, their susceptibility to pathogens is even higher, and pathogens that would normally be subclinical can become clinical.

We were able to minimise the effects of stress and NSAIDs on the gastric mucosa and diagnose and treat anorexia related to gastrointestinal infections. Hence, other incidences of anorexia were most probably linked to pain, which was misdiagnosed during the study and could be managed in future studies with appropriate prophylactic measures.

4.2 Study design

Next to the most suitable animal, an appropriate design has to be chosen for a study. Therefore, the more a specific study design is established, the less likely major mistakes are done and different studies are easier to compare.

Studies with acute defects (like Part A) around implants are described only rarely in minipigs [120, 127]. Takasaki *et al*. created acute defects on the buccal aspect of the implant surrounding bone. After a healing period, an inflammation was provoked and treated with laser [120]. In Part A, the non-infected defects were supposed to be less challenging for the test item compared to the infected Part B. It was believed, that the environment could be controlled, and a spontaneous progression of bone loss would not occur. However, this goal was not reached in the end – most probably because of wound dehiscence. A severe peri-implantitis developed including bone loss and soft tissue inflammation.

The ligature inducing peri-implantitis in Part B was designed to resemble a naturally occurring disease. Even though being described in minipigs only rarely [111, 116, 119], induction of peri-implantitis with ligature is a well described procedure in dogs. At test item application, peri-implantitis including bone loss and soft tissue inflammation was provoked.

At sacrifice, Part A presented itself with a more severe peri-implantitis situation including soft tissue inflammation and possible bone resorption (Tab 7.1.3). Initially though, Part A was designed to be less severe compared to Part B. Part A defects were created in a clean and noninflamed environment, but in non-osseointegrated implants. In Part B on the other side, defects developed due to inflammatory bone resorption and inflammation of soft tissue in osseointegated implants. Hypothetically, a 6 weeks healing period after TI application in Part A would ideally result in more bone formation than a 4 weeks period in Part B. On the other side, larger buccal defects of Part A might need longer to heal completely than smaller circumferential defects in Part B. Furthermore, a possible negative influence of soft tissue healing after an additional surgical procedure as in Part B remains unknown. Hence, both study parts cannot be compared as they are designed to answer different questions. If one would like to compare the two parts of the study, same sized defects would have to be created in osseointegrated implants. In addition, the timeline between test item application and sacrifice would have to be the same. Which design was the more challenging in the end, is difficult to state retrospectively, even though it would be of interest for future studies.

For the assessment of the efficacy of a test item, it is important to compare the TI results with a control group. In the present study, the TI sites and Ctr sites were in the same animal, because, animals show very individual reactions during the studies. To separate test item and control sites, a split-mouth design was chosen. Hence, the TI could not influence the adjacent Ctr sample. With this design, however, it was not possible to make any statement regarding the toxicity and systemic influences of the evaluated material because no animals were treated only with Ctr.

Nevertheless, blood samples were taken and checked for any severe reactions in response to the interventions and as an indication of the animals' systemic health. According to the author's knowledge, peri-implantitis does not result in a systemic infection and therefore alteration in blood values are usually not seen. Additionally, linking specific alterations in blood results to the administration of test item, is not possible with this study design,

particularly when events such as surgery, pain, infections unrelated to the study may alter blood values as well. Nevertheless, serum samples before and after test item administration were collected and stored for potential analysis in the future. To increase the value of these findings in the future, a small animal control group, that is not treated with TI at all, is needed. In such a control group, blood sampling has to be carried out in the same time intervals as it was done in the present study. Furthermore, a clinical normal range for individual blood parameters in Ellegaard Göttingen minipigs is not described, but only average values, which makes the detection of clinical deviations from baseline values difficult.

Congruent to the blood results, the extracted soft tissue samples were not processed histologically but simply stored in 4% formalin for future analysis. This was done because of limited present value and limited funds. Similar to the blood samples, the samples can be processed, and compared to those collected from a non-treated reference animal in the future. If so, soft tissues from this animal would act as a baseline to provide the appropriate reference when evaluating toxicity of a test item.

Additional radiographs taken during an anorectic phase of one animal (85.05) revealed that teeth roots were not totally extracted. According to the surgeon, the complete cleaning of the tooth sockets was not possible in this animal. It was decided to leave the remnants of the roots inside. However, in histology, it was discovered, that the 85.05 L4 implant was placed into a root remnant and osseointegration did not occur in this area. This might have influenced the initial healing period as well as the healing period after TI application negatively. On the other hand, in the comparison of the results of 85.05 L4 (Overall score 7, TI1: mean: 9.67) with other samples, it did not perform worse. Hence, the impact of remaining parts of roots is questionable. It seems to be more problematic, that all number one implants were placed into the root of the canine. Thus, radiographs to control the absence of root remnant and a correct placement of implants, are highly suggested in future studies and is also done by other groups [127].

4.3 Implant dimension

In the present study, the correct positioning of the implants turned out to be challenging. This resulted in 16/16 of position 1 (most mesial) implants placed at least partially into the root of the canine. As a consequence, these samples were excluded from histologic

evaluation and most of their radiographs could not be evaluated. Macroscopically, these implants performed worse compared to the other positions (see Fig 4.1).

Position	Part A	Part B
1	10.88 ± 2.85	8.13 ± 1.73
2-4	11.29 ± 2.42	6.08 ± 1.44

Tab. 4.1: Position 1 compared to 2-4 in macroscopic evaluation (0-21; 0 being the best score) for Part A and Part B

In histology, the tip of some implants almost reached the alveolar canal. Placing implants into a tooth or its root or the alveolar canal might influence its osseointegration and therefore produces bias.

Authors reported implants penetrating the canine root before (Tab. 4.2). In a study by Stadlinger *et al*., male and female minipigs (age: 12 months) were used in an osseointegration model. All animals had 3 implants placed in each mandible, with only the primary premolars extracted. The group used implants with a length of 12.00 mm and a diameter of 4.25 mm. They found that a relevant number of implants were placed into the root of the canine. Furthermore, they encountered the problem of implants penetrating the alveolar canal [128]. Nkenke *et al*. on the other hand, used implants with a length of 11 mm and a diameter of 3.8 mm in 12 months old minipigs. In order to investigate the influence of direct load on the implants, they placed five implants adjacent to each other in the mandible of a partially edentulous minipig. The group did not observe implants in the canine nor in the alveolar canal [129]. As well as Nkenke *et al*., other authors did not have this problem. Stübinger *et al.* reevaluated the ligature-induced peri-implantitis model in 1.4 years old male minipigs. Four implants of 8mm in length and a platform diameter of 4.0 mm were placed in a partially edentulous Göttingen minipig. No implant was placed into the canine [119].

Author	Gender F:M	Age (months)	Number of implants per side	Implant length (mm)	Implant diameter (mm)	Alveolar canal	Root of canine
Nkenke et al (2003)	7:1	18-21	5	12.00	4.25	No	No
Stadlinger et al (2008)	10:10	12	3	11.00	4.25	Yes	Yes
Stübinger et al (2016)	0:6	15	4	8.00	4.00 (platform)	No	No
Present study	8:0	22-24	4	10.00	4.8 (platform)	No	Yes

Tab. 4.2: Table comparing different study with dental implants in minipigs according animal and implant specifications and whether the alveolar canal or the root of the canine is penetrated F: female; M: male; Length and diameter in mm

The size of the root of the canine seems to be individually different and depending on age and gender. But, to the author's knowledge, no published data is available investigating the differences of male and female canines in minipigs. Nevertheless, in direct comparison, the canine in males appears steeper than in female. Hence, it is believed, that the root in females is more towards the bone crest. According to Stadlinger *et al*., the root of the canine might be larger in male pigs and, therefore, the group proposed to take female pigs for such studies only. Even though Nkenke *et al*. did not have these problems with longer implants, it can be suggested that longer implants rise the risk of penetrating the alveolar canal or the canine root dramatically.

Radiographs performed prior to implantation to check individual dimensions of the canine root can significantly reduce the risk of implant misplacement. In addition, we suggest, that, when operating minipigs, shorter implants with a maximal length of 8.00 mm are used and the distal area of the canine is avoided.

Another aspect that has to be kept in mind, is the interspace between neighboring implants. But the available information on implant and abutment dimensions is restricted in most ligature-induced peri-implantitis studies in minipigs [116, 118]. The group around Stübinger *et al.* gave full information on dimensions of implants and abutments but no information on interspaces. The group used significantly smaller abutments (platform: 4 mm) than the ones from the present study (platform: 5.5 mm) [119]. The interspace is important to ensure the integrity of adjacent implants. In addition, osseointegration, soft tissue healing and the test item might be influenced, when placed too close to each other. When abutments are used to create a ligature induced circumferential peri-implantitis, an even larger interspace must be considered. To the authors knowledge, there is one study

investigating the interspace between implants only, but, unfortunately, there is no study regarding the minimum distance between abutments. Tarnow *et al*. suggest a minimum 3 mm between implants [130]. If abutments are used, we suggest the interspace should exceed the diameter of the abutment at least 2 mm.

To consider the 3Rs, a cadaver test on dead animals was carried out in advance to the study. Due to restricted availability of miniature pigs, a domestic pig was used instead. Given anatomical differences between domestic and miniature pig, four implants might have fitted well in the mandible of the domestic pig, but this did not track to minipigs.

It is strongly debatable if four implants with a platform diameter of 5.5 mm can be placed next to each other and still respecting the suggested interspaces. With maximally 2 implants placed per side, interspaces could be maintained and most probably, the most mesial implant could be placed further away from the canine.

4.4 Dehiscence

Two different strategies can be chosen for successful osseointegration of implants and healing of the surrounding mucosa: transmucosal and submucosal. In transmucosal healing, abutments are placed on the implants and reach through the mucosa. In this case, the mucosa acts as a seal around the abutments. Good results of transmucosal healing can be seen in healthy mucosal tissue. If a submucosal healing strategy is chosen, the mucosa is closed on top of the implants which creates a protected microclimate under the mucosa and is therefore protected from external affection [131]. Because transmucosal healing works best in healthy mucosal tissue (not the case in Part B) and influences arising from the oral cavity wanted to be held down, submucosal healing was chosen - like in most of the peri-implantitis studies [106].

Unfortunately, submucosal healing could not be obtained, and wound dehiscence turned out to be one of the major difficulties in the interpretation of the results because of possible test item displacement and progression of peri-implantitis. Right from the surgeries on, the tension on the mucosa and the suture material seemed high when closing the mucosal flap. In one animal (85.08) a mucosal flap with rotated sublingual tissue had to be performed for complete closure which shows the high tension. Despite the fact, that mucosa had to be transpositioned only once (85.08 left), all implants of Part A and Part B were exposed to the oral cavity at sacrifice. Some were half covered with mucosa, but in most implants, the cover screws were fully visible. Hence, the question of timepoint and reason of dehiscence arises and then, also the possible timepoint of test item

displacement. It acts on the assumption, that peri-implantitis progressed after eruption of the heads of the implants in both part A and B. This would be congruent with the observation of Albouy *et al.* in dogs [115]. On one side, it would underline the similarity to naturally occurring peri-implantitis in humans, but the expected healing of the created defects has most probably not occurred.

In peri-implantitis studies, dehiscence is a known problem. Hale *et al*. introduced the minipig as a model for osseointegration in 1991. After a post extraction healing phase, the group placed 10 dental implants. Healing was supposed to be submucosal. But within one week, all of the implants were exposed to the oral cavity without a covering mucosa. They observed superficial bone loss, but osseointegration in the deeper areas [132]. Even though deeper areas where not evaluated for the present study, most implants seemed to be osseointegrated in deeper areas.

Olsen *et al*. faced similar problems. The purpose of the study was to evaluate bone augmentation material in the minipig. Two implants per side were placed, defects were created, augmented and covered with a collagen membrane. Two weeks after test item application, implants were exposed, the membrane was lost and so were the bone grafts. The group interpreted the reason being unwanted weight load on suture and implant from the tongue and food [133].

During the present study, animals were held on a bedding mixed of wood chips and straw. The pigs were observed chewing on the straw as an activity. Straw was left inside as an enrichment of the environment and for animal welfare. With its sharp ends, the straw might well have contributed to early dehiscence. Straw irritates the mucosa and also the suture material.

But more importantly, it is possible, that tension on the mucosa and its suture material was too high and dehiscence was the natural consequence. For the implants used in the present study, the manufacturer gives two guidelines for positioning. The implants are either placed 1 mm or 2 mm supracrestal. In the present study, the implants were placed in the 2 mm position. In hindsight, the 1 mm position might have been the better option, because the forces on the suture material and mucosa would have been lower. Furthermore, bone level implants may have been an option as well. The advantage is, that there is no tension at all from the inside on the suture. In bone level implants, heads of the implants will be exposed through the lowering of the bone crest. This exposure should provide a sufficient area for TI placement.

Using Barrier membranes is preferred by many dentists even though the benefits are not proven [23]. When testing new materials, one would like to have as few other materials than the test item as possible. Materials might interact with the test item or might influence the outcome of the study. In the present study, a membrane was still chosen for several reasons: it was meant to keep the test item on site and protect the defects from epithelial ingrowth. Furthermore, we wanted to stay as close as possible to naturally occurring peri-implantitis and its clinical treatment.

As a membrane, the Reguarde™ resorbable membrane by Implant Direct (Switzerland) was chosen. It is derived from bovine collagen type 1. Another choice would have been the Bio-Gide® membrane manufactured by Geistlich, Switzerland, made out of porcine derived collagen type 1 and 2. According to the surgeon, who was more familiar with the Bio-Gide® membrane, pointed out some differences. The Reguarde™ seemed stiffer and thicker to him. Unfortunately, no information is provided by the manufacturer regarding the thickness of the membrane. The stiffness made it harder for the surgeon to adapt the membrane correctly. The challenging adaption might be another component, that helps to explain the events occurring during the course of the study. In this case, we believe, that the membrane might have ground at the suture and contributed to the wound dehiscence. Another fact is, that in all animals the membrane was fixed using the cover screw 1 and 4, except of animal 85.01 right side. Most cover screws that were loose or lost, were lost in these positions (Tab. 7.1.3, Tab. 7.1.4). The right sided cover screws of 85.01 were still on site and tight. It might be possible, that loose cover screws stimulated the suture and progressed dehiscence.

Macroscopically, the membrane could not be detected in any sample. Due to the fact, that the membrane could neither be found macroscopically nor microscopically, it must have been lost during their in-life period. According to the manufacturer, the Reguarde™ membrane has a in vivo resorption of 26 to 38 weeks. If not lost, it must have been seen. The membrane could not stop epithelial ingrowth into the implant/soft tissue cavity and it also did not prevent the test item from displacement. It is strongly debatable if the membrane we used, had a positive contribution to the study.

4.5 Bone resorption and formation

Bone resorption and formation were supposed to be key elements in the evaluation of the results for the study. Defects were created and ought to minimize in TI treated sites compared to control sites. Hence, measurements of the mandibular bone were done at

several timepoints: application of the test item and post mortem on CT images and on histology images. All measurements were done on the buccal aspect of the bone. Unfortunately, bone resorption of the buccal aspect could not be measured on radiologic images, because implants were overlapping with the radiolucent zone. In addition, measuring depth and bone crest could not be performed during the macroscopic evaluation at sacrifice because the bone was covered with mucosa and mucosa was preserved for histological evaluation. Only the extent of implant exposure was determined at this point.

Measurements of the defects during surgery were done using a calliper. This measurement device has a lack of accuracy - especially in small sized defects and it was not possible to measure the pocket depth with the device. A constant measurement point was critical to determine in all measurements. Also, on CT images and in histology, because cover screws were not always tightened enough, but if they were tight, no borderline between implant and cover screw could not be seen.

Because of the usage of different measurement tools, measurements could only be compared in between the same measurements type (CT or histology) but not across different methods.

Hence, for Part A only in-surgery and CT measurements were available. In Part B additional and more accurate histology measurements were available.

The accuracy of the measurements in the present study is questionable and needs to be standardised for future studies. Commercially available probing devices would be suggested for in-life measurements and if there is evidence of cover screw loosening, screws should be removed prior to measurements. Hence, also the value of the measurements is not as clear as expected. The comparison of defect sizes might be of higher value, when compared individually, than between all animals.

4.6 RRR (Replacement, reduction, refinement)

Since many years, there is scientific interest to improve animal models while at the same time a societal interest to reduce the number of animal trials. The term of the 3R’s was introduced by Russel in 1959: Reduce the number of animals used in a study, replace the animals by either other animals or various biological systems and to refine study design so less animals are needed [134]. Several steps were done to meet the demands of the 3 R’s within the present study.

Ahead of the study, a cadaver test was carried out. The cadaver test and surgeries within the study were carried out by the same experienced maxillofacial surgeon who has operated the same animal model before. An experienced surgeon and additional training in the cadaver test, reduced the risk of surgery related production of bias.
Unfortunately, due to limited availability, the tests were carried out in a pig (*Sus scrofa domesticus*) instead of using the same animal breed as in the study. Furthermore, the barrier membranes used in the study were not available for the cadaver test, hence the surgeon could not give his opinion on the stiffness in advance. Furthermore, it is very likely, that too many implants were placed on each side. While this was a measure to reduce animal numbers by having multiple implant sites to evaluate within one animal, as a consequence however, implants could not be evaluated and furthermore, all other specimen did not show the expected results. In future studies, cadaver tests are best carried out in the same breed. Thus, tightness of the mucosa and the narrowness between the implants could have been noticed and the number of placed implants could have been reduced. Additional radiographs or CT images could have been taking during a cadaver study to confirm suitable implant dimensions and numbers and, also, the correct placement.
Due to several reasons, the current study design was very challenging to test a gel, that was only tested in rat femoral defects before. In future studies, it could be tested in in a step by step process, starting in local, non-infected drill hole defects in the ovine hip, femur or humerus as described by our group before [111, 135]. Same implants and barrier membranes could be used to reveal possible influences with the testing system. These ovine models could also be done with infected defects resembling a peri-implantitis study without external influences such as a tight mucosa or chewing by the animals. If successful, a more challenging study like the present study could be carried out with an improved implant design.
In another approach, the same study design could have been used, but carried out in stages. In such a design, the animal model could have been improved after the first one or two animals (e.g. radiographs, number of implants, defect measurements) were included in the study.

However, the present study was designed as a pilot study with only 4 animals in each group. With a little number of animals, we were able to receive profound information on challenges of such a study and possible improvements for future studies.

In addition, it was shown again, that minipigs would be a powerful animal model for investigations on peri-implantitis studies if adequate improvement of the model is carried out.

4.7 Gel

A gel containing an osteoconductive material which was shown to have additional osteoinductive abilities like hydroxyapatite is supposed to reduce defect sizes by mineralizing and creating a starting point for bone formation. The efficacy of hydroxyapatite was shown before in several studies [71, 80, 136]. Furthermore, previous studies have indicated, that a gel with hyaluronic acid, HA and BP shows rapid mineralisation already within 30 days [137].

A rise of the bone crest, smaller defects, or mineralised structures, were expected to be observed in the present study. Instead, a picture of eroded implants and large bone defects was seen. Macroscopically, the superficial search for remnants of TI or membrane was negative in all specimen. To keep the soft tissue intact for a complete histologic evaluation including the soft tissue, the evaluator refrained from searching for remnants of the TI deeper in the tissue. But also, the microscopic observations could not reveal any remnants of the TI in any of the animals. For Part A, histology processing did not proceed beyond the preparation of several representative samples. No new insights were expected from the preparation of more histological slides. The histology slides of Part B were compared to different embedded and stained samples of the TI alone or its appearance in the subcutis of a rat. But no similar structures could be detected in any of the slides of Part A and Part B. Also, no mineralised structure like those described in other studies could be detected, which was expected to happen early in the healing phase [138].

Bisphosphonates have a strong affinity to bind to HA particles and build complexes. If metabolised by osteoclasts, bisphosphonates will be set free and provoke apoptosis of osteoclasts. Hence, the bisphosphonates are released only passively from the first day on and are depending on ingrowing soft tissue and cell population to propagate their activity. That makes it difficult to predict the speed of action of bisphosphonates. However, there is a positive trend seen in the TI2 group in which bisphosphonates seem to be able to reduce the pocket size. On the other side, if the gel did not stay in place for a long time, the question arises how to explain the slightly lesser bone resorption in TI2 sites. It is likely possible, that after debridement, osteoclasts remained on site and they could have

taken up the HA-BP complexes and provoked apoptosis. Another possibility is, that not all BPs were bound to HA in the gel and some was still free to bind to form complexes with free, endogenous HA. During bone resorption processes, these complexes could have been taken up as well and provoked apoptosis of osteoclasts.

Unfortunately, due to the small number of implants, a statistical statement is not possible, and peri-implantitis progressed after TI was washed away, but may have left behind a situation that is a little bit more stable.

Previous studies with the same hydrogel and same composition, show good osseoinductive abilities in the femur of a rat. The same group were able to show, that hyaluronic acid is fully degraded only after 60 days. Mineralisation should have its peak during the first 30 days in the TI2 group. Without bisphosphonates most of the bone resorption is seen between day 10 and 31 [97].

It was concluded, that the gel was most likely displaced, before being mineralised. A gel that is injected, needs several properties. First of all, the gel has to be soft, so it can be injected around the area and fill up space, that would remain dead space if filled up with blocks of biomaterial. On the other side, if the viscosity is too low, it might be displaced easier than a gel with higher viscosity. Animals, such as minipigs, tend to chew on various items (e.g. straw, grid) for pastime. In any case, also a faster mineralisation of the gel would have helped the gel to stay on site and is desired if placed on areas, where forces are applied on, like the oral cavity.

It is problematic, that the timepoint of dehiscence remains unknown in the animals. But as peri-implantitis progressed after the mucosa opened up, the situation became more challenging for the gel and possible mineralisation or even bone formation in such circumstances are doubtful.

4.8 Conclusion

The results of Ctr and TI are very close in both parts of the study. Only in Part B, there is a slight trend for TI2 to be beneficial in the treatment. However, many unknown factors, such as an unknown membrane, mal-positioning or dehiscence make a distinguished statement on the efficacy of the TI impossible. The animal model as performed did not create a controlled peri-implant defect situation or peri-implant healing situation. Instead, many complications compounded by the inability of TI to stay in place made it too challenging to differentiate between Ctr and TI. Reducing the number of animals and increasing the number of tested specimen per animal at the same time, is not beneficial

or in compliance with 3R principles, if results cannot be interpreted. A better balance must be found for future studies to create controlled per-implant defect situations and build the study step by step with smaller pilots.

5 Literature

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6 Abbreviations

3R	Replace Reduce Refine
ATP	Adenosin-Tri-Phosphate
BID	Bis in die (twice a day)
BP	Bisphosphonate
BW	Bodyweight
CT	Computertomography
Ctr	Control
EDTA	Ethylenediaminetetraacetic acid
ePTFE	Expanded polytetrafluoroethylene
i.m.	Intramuscularly
i.v.	Intravenously
kV	Kilovolt
L	Left
mAS	Milliampere second
(P)MMA	(Polymerized-) Methyl methacrylate
(n)HA	(Nano-) Hydryxyapatite
n.a.	Not applicable
NSAID	Non-steroidal anti-inflammatory drug
p.o.	orally
R	Right
RBS	Rapid Bone Staining
SID	Semen in die (once a day)
TCP	Tricalcium phosphate
TI1	Test item 1
TI2	Test item 2

7 Appendix

7.1 Tables

Implant / Animal	R2	R3	R4	L2	L3	L4
85.01	O	✓	O	O	✓	O
85.02	✓	✓	O	O	✓	✓
85.03	O	O	O	O	O	O
85.04	O	✓	✓	✓	✓	✓
85.05	✓	✓	✓	✓	✓	✓
85.06	✓	✓	✓	✓	✓	✓
85.07	✓	✓	✓	✓	✓	✓
85.08	✓	✓	✓	✓	✓	✓

Tab. 7.1: Implants that were processed for histologic evaluation. "✓" means processed; "O" means not processed. No number 1 implants were processed.

Part A

Group	Animal	Implant	Depth (mm)	Width (mm)
Ctr	85.01	R1	7	4
	85.01	R2	5	3
	85.01	R3	4	4
	85.01	R4	5	4
	85.02	L1	5	5
	85.02	L2	5	5
	85.02	L3	5	5
	85.02	L4	5	5
	85.03	R1	6.5	5
	85.03	R2	5	5
	85.03	R3	5	6
	85.03	R4	5	5
	85.04	L1	5.5	5.5
	85.04	L2	5.5	5
	85.04	L3	5	5
	85.04	L4	5	5
TI1	85.01	L1	5	4.5
	85.01	L2	5	4.5
	85.01	L3	4.5	5
	85.01	L4	5	5
	85.02	R1	5.5	5
	85.02	R2	6	5
	85.02	R3	5	5
	85.02	R4	5	5
TI2	85.03	L1	5.5	5
	85.03	L2	5	5
	85.03	L3	5	5.5
	85.03	L4	5	5
	85.04	R1	5.5	5
	85.04	R2	5	5
	85.04	R3	5	6
	85.04	R4	5	5

Part B

Group	Animal	Implant	Depth (mm)	Width (mm)
Ctr	85.05	R1	3	n.d.
	85.05	R2	4	n.d.
	85.05	R3	3	n.d.
	85.05	R4	2	n.d.
	85.06	L1	3.5	n.d.
	85.06	L2	3	n.d.
	85.06	L3	3	n.d.
	85.06	L4	2.5	n.d.
	85.07	R1	3	n.d.
	85.07	R2	3	n.d.
	85.07	R3	2	n.d.
	85.07	R4	2	n.d.
	85.08	L1	3.5	n.d.
	85.08	L2	3.5	n.d.
	85.08	L3	2.5	n.d.
	85.08	L4	3	n.d.
TI1	85.05	L1	4	n.d.
	85.05	L2	4	n.d.
	85.05	L3	3	n.d.
	85.05	L4	3	n.d.
	85.06	R1	3	n.d.
	85.06	R2	4	n.d.
	85.06	R3	3	n.d.
	85.06	R4	3	n.d.
TI2	85.07	L1	2	n.d.
	85.07	L2	3	n.d.
	85.07	L3	3	n.d.
	85.07	L4	3	n.d.
	85.08	R1	3	n.d.
	85.08	R2	4	n.d.
	85.08	R3	3	n.d.
	85.08	R4	2.5	n.d.

Tab. 7.2: The tables summarize the measured defect sizes right before test item implantation. The defects were measured in mm. In Part B the width could not be measured (indicated with n.d.), because neighbouring defects could not be separated

Part A

Group	Animal	Implant	Redness (0 - 3)	Swelling (0 - 2)	Surface (0 - 2)	Inflammation (0 - 3)	Breach (0 - 3)	Hyperplasia (0 - 2)	Firmness (0 - 2)	Cover screw (0 - 2)	Interspace (0 - 2)	Total (0 - 21)
Ctr	85.01	R1	3	0	2	3	3	0	0	0	1	12
	85.01	R2	3	0	2	3	3	0	2	0	2	15
	85.01	R3	3	0	2	3	3	0	2	0	2	15
	85.01	R4	3	0	2	3	3	0	2	0	2	15
	85.02	L1	2	0	1	2	1	0	0	0	1	7
	85.02	L2	3	0	1	3	2	0	0	0	1	10
	85.02	L3	3	0	1	3	2	0	0	0	1	10
	85.02	L4	2	0	1	2	2	0	0	1	0	8
	85.03	R1	3	0	2	3	2	0	0	1	0	11
	85.03	R2	3	0	2	3	2	0	0	0	1	11
	85.03	R3	3	0	2	3	2	0	2	0	1	13
	85.03	R4	2	0	1	3	2	0	0	1	0	9
	85.04	L1	3	0	1	3	3	0	2	2	0	14
	85.04	L2	3	0	1	2	2	0	0	0	2	10
	85.04	L3	3	0	1	2	2	0	0	0	2	10
	85.04	L4	2	0	1	2	1	0	0	2	1	9
TI1	85.01	L1	3	0	2	3	3	0	0	2	1	14
	85.01	L2	3	0	2	3	3	0	0	2	1	14
	85.01	L3	3	0	2	3	3	0	0	0	2	13
	85.01	L4	3	0	2	3	3	1	2	2	2	18
	85.02	R1	3	0	2	2	3	0	0	1	1	12
	85.02	R2	3	0	2	2	3	0	0	0	1	11
	85.02	R3	3	0	2	2	3	0	0	0	0	10
	85.02	R4	2	0	2	2	2	0	0	0	0	8
TI2	85.03	L1	2	0	1	1	1	0	0	0	1	6
	85.03	L2	3	0	2	2	2	0	0	0	2	11
	85.03	L3	3	0	2	2	2	0	0	0	2	11
	85.03	L4	2	0	1	1	1	0	2	1	1	9
	85.04	R1	3	0	1	2	3	0	2	0	2	13
	85.04	R2	3	0	1	2	3	0	0	0	2	11
	85.04	R3	3	0	1	2	3	0	0	0	1	10
	85.04	R4	3	0	1	2	3	0	0	1	1	11

Fig. 7.3: The table summarizes the macroscopic evaluation of Part A for each implant including redness, swelling surface, Inflammation, breach, hyperplasia, firmness, cover screws, interspace and gives a total of points (0-21; 0 being the best score).

Part B

Group	Animal	Implant	Redness (0 - 3)	Swelling (0 - 2)	Surface (0 - 2)	Inflammation (0 - 3)	Breach (0 - 3)	Hyperplasia (0 - 2)	Firmness (0 - 2)	Cover screw (0 - 2)	Interspace (0 - 2)	Total (0 - 21)
Ctr	85.05	R1	3	0	1	2	2	0	0	0	0	8
	85.05	R2	2	0	1	2	2	1	0	0	0	8
	85.05	R3	1	0	1	1	2	0	0	0	0	5
	85.05	R4	1	0	1	1	1	2	0	0	0	6
	85.06	L1	3	0	1	2	3	0	0	0	0	9
	85.06	L2	2	0	1	2	3	0	0	0	0	8
	85.06	L3	1	0	1	1	2	0	0	0	0	5
	85.06	L4	2	0	1	2	2	0	0	0	0	7
	85.07	R1	1	0	1	1	2	0	0	0	0	5
	85.07	R2	2	0	0	2	2	0	0	0	2	8
	85.07	R3	2	0	0	1	2	0	0	0	2	7
	85.07	R4	1	0	0	1	2	0	0	0	1	5
	85.08	L1	3	0	1	2	3	0	0	2	0	11
	85.08	L2	1	0	1	2	2	1	0	0	0	7
	85.08	L3	2	0	1	3	2	0	0	0	0	8
	85.08	L4	1	0	1	2	2	1	0	0	0	7
TI1	85.05	L1	3	0	1	2	2	0	0	0	0	8
	85.05	L2	2	0	1	2	2	0	0	0	0	7
	85.05	L3	1	0	1	1	2	0	0	0	0	5
	85.05	L4	1	0	1	1	2	0	0	0	0	5
	85.06	R1	3	0	1	2	2	0	0	0	0	8
	85.06	R2	3	0	1	2	2	1	0	0	0	9
	85.06	R3	2	0	1	2	2	0	0	0	0	7
	85.06	R4	1	0	1	1	2	0	0	0	0	5
TI2	85.07	L1	3	0	0	1	2	1	0	0	0	7
	85.07	L2	1	0	0	2	1	0	0	0	0	4
	85.07	L3	1	0	0	1	1	1	0	0	0	4
	85.07	L4	2	0	0	1	2	0	0	0	0	5
	85.08	R1	3	0	1	2	3	0	0	0	0	9
	85.08	R2	2	0	1	2	2	0	0	0	0	7
	85.08	R3	1	0	1	1	2	0	0	0	0	5
	85.08	R4	2	0	1	2	2	0	0	0	0	7

Fig. 7.4: The table summarizes the macroscopic evaluation of Part B for each implant including redness, swelling surface, Inflammation, breach, hyperplasia, firmness, cover screws, interspace and gives a total of points (0-21; 0 being the best score).

Part A

Group	Animal	Implant	Radiolucent zone (0-3)	Bone resorption interspace (0-3)	Sclerotic rim (0-3)	Total (0-9)
Ctr	85.01	R1	1	2	0	3
	85.01	R2	2	2.5	0	4.5
	85.01	R3	3	3	0	6
	85.01	R4	3	3	0	6
	85.02	L1	n.d.	1	n.d.	n.a.
	85.02	L2	2	1	n.d.	n.a.
	85.02	L3	2	0.5	0	2.5
	85.02	L4	3	0	0	3
	85.03	R1	2	0	0	2
	85.03	R2	2	1	0	3
	85.03	R3	3	2.5	0	5.5
	85.03	R4	3	3	0	6
	85.04	L1	n.d.	1	0	n.a.
	85.04	L2	2	1.5	0	3.5
	85.04	L3	2	2	0	4
	85.04	L4	3	2	0	5
TI1	85.01	L1	2	2	0	4
	85.01	L2	2	2	0	4
	85.01	L3	2	2.5	0	4.5
	85.01	L4	3	3	0	6
	85.02	R1	2	1	0	3
	85.02	R2	3	1	0	4
	85.02	R3	2	1	0	3
	85.02	R4	3	1	0	4
TI2	85.03	L1	2	2	0	4
	85.03	L2	2	2.5	0	4.5
	85.03	L3	2	3	0	5
	85.03	L4	3	3	0	6
	85.04	R1	2	1	0	3
	85.04	R2	1	1.5	0	2.5
	85.04	R3	3	2.5	0	5.5
	85.04	R4	3	3	0	6

Part B

Group	Animal	Implant	Radiolucent zone (0-3)	Bone resorption interspace (0-3)	Sclerotic rim (0-3)	Total (0-9)
Ctr	85.05	R1	n.d.	0	0	n.a.
	85.05	R2	1	0	0	1
	85.05	R3	0	0	1	1
	85.05	R4	2	0	1	3
	85.06	L1	2	0	0	2
	85.06	L2	2	0	0	2
	85.06	L3	2	0	1	3
	85.06	L4	0	0	1	1
	85.07	R1	n.d.	0	0	n.a.
	85.07	R2	0	0	0	0
	85.07	R3	0	0	0	0
	85.07	R4	1	0	0	1
	85.08	L1	2	1	0	3
	85.08	L2	1	0.5	0	1.5
	85.08	L3	0	0	0	0
	85.08	L4	0	0	0	0
TI1	85.05	L1	2	0	0	2
	85.05	L2	1	0.5	0	1.5
	85.05	L3	2	1.5	0	3.5
	85.05	L4	2	2	0	4
	85.06	R1	1	0	0	1
	85.06	R2	1	0	0	1
	85.06	R3	2	0	1	3
	85.06	R4	1	0	1	2
TI2	85.07	L1	n.d.	1	0	n.a.
	85.07	L2	2	0.5	0	2.5
	85.07	L3	0	0	0	0
	85.07	L4	0	0	0	0
	85.08	R1	0	0	0	0
	85.08	R2	0	0	0	0
	85.08	R3	1	0	0	1
	85.08	R4	1	0	0	1

Tab. 7.5: The tables show the results of the radiologic evaluation. For each implant, the radiolucent zone, the interspace between bone resorption and the sclerotic rim were evaluated, scored and summarized in a total number of points (0-9; 0 being the best score). If the radiolucent zone could not be evaluated, score was "n.d." and not total score was calculated.

Group	Animal	Implant	Soft tissue thickness (µm)	Epithelium invasion (µm)	Pocket width (µm)	Implant-Bone crest (µm)	Pocket depth (µm)	Inflammation (0-3)	Bone activity-Buccal (0-2)	Bone-activity Lingual (0-2)	Total (0-7)
Ctr	85.05	R2	358	276	18	181	17	2	0	2	4
	85.05	R3	251	261	139	95	156	1	2	2	5
	85.05	R4	252	218	51	47	43	2	0	1	3
	85.06	L2	504	390	0	75	0	3	2	2	7
	85.06	L3	354	442	120	144	99	1	0	1	2
	85.06	L4	275	206	11	127	38	2	1	2	5
	85.07	R2	370	233	45	143	31	1	0	1	2
	85.07	R3	291	229	0	74	0	1	0	1	2
	85.07	R4	206	148	65	61	103	0	0	1	1
	85.08	L2	414	173	38	250	21	2	0	1	3
	85.08	L3	530	467	0	195	0	1	1	1	3
	85.08	L4	388	227	0	172	0	2	0	1	3
TI1	85.05	L2	325	347	0	146	0	1	1	2	4
	85.05	L3	188	221	87	107	36	1	2	1	4
	85.05	L4	292	271	42	128	60	0	1	1	2
	85.06	R2	478	378	0	197	0	0	1	1	2
	85.06	R3	402	241	0	155	0	0	1	2	3
	85.06	R4	359	285	67	125	59	1	2	2	5
TI2	85.07	L2	458	610	187	178	556	3	2	1	6
	85.07	L3	453	377	0	111	0	1	0	0	1
	85.07	L4	378	359	0	125	0	0	0	0	0
	85.08	R2	437	293	63	270	65	1	0	2	3
	85.08	R3	222	165	0	92	0	1	0	2	3
	85.08	R4	184	114	0	185	0	1	1	2	4

Tab. 7.6: The table summarizes the histologic evaluation including measurements of the soft tissue thickness, epithelial invasion, pocket width, implant-bone crest distance, and the pocket depth (measurements were done in µm). Furthermore, inflammation, bone activity on the buccal and on the lingual of the implant were scored and a total score was calculated (0-7; 0 being the best score)

7.2 Figures

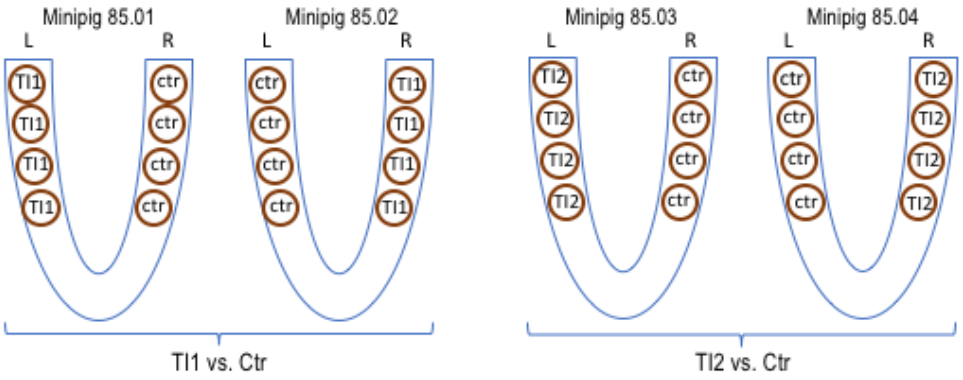

Fig. 7.1: Part A: study design; each circle represents one implant. "ctr" indicates, that defects were left empty, TI1 and TI2 indicates that defects were treated with the particular test item.

7.3

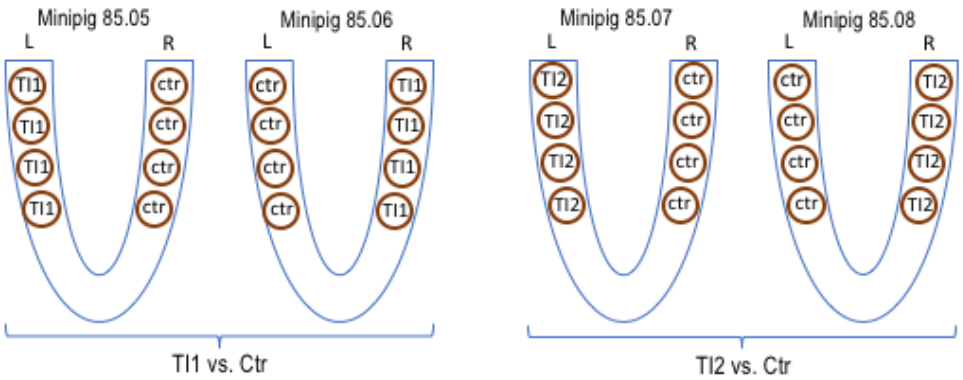

Fig. 7.2: Part B: study design; each circle represents one implant. "ctr" indicates, that defects were left empty, TI1 and TI2 indicates that defects were treated with the particular test item.

Fig. 7.3: Figure shows the numbering of the implants. Number 1 is the most mesial implant and number 4 the most distal one. “L” is left and “R” is right

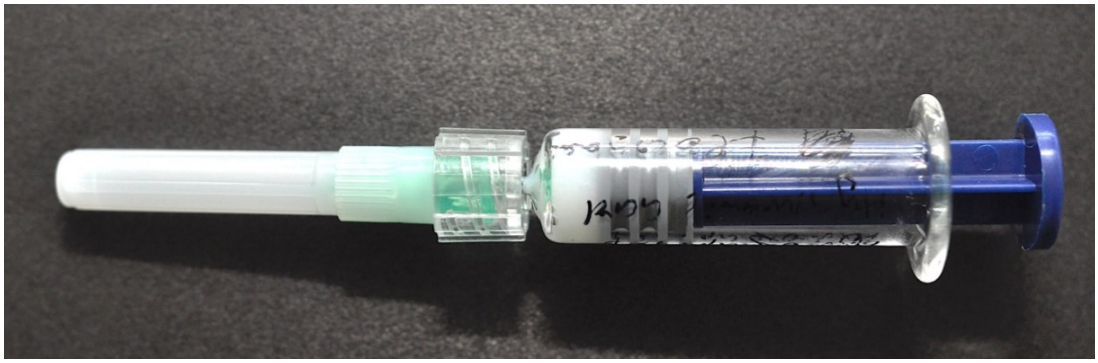

Fig. 7.4: Ready to use applicator with a 21G canule. There is still test item in the syringe (white). It was delivered sterilized and wrapped.

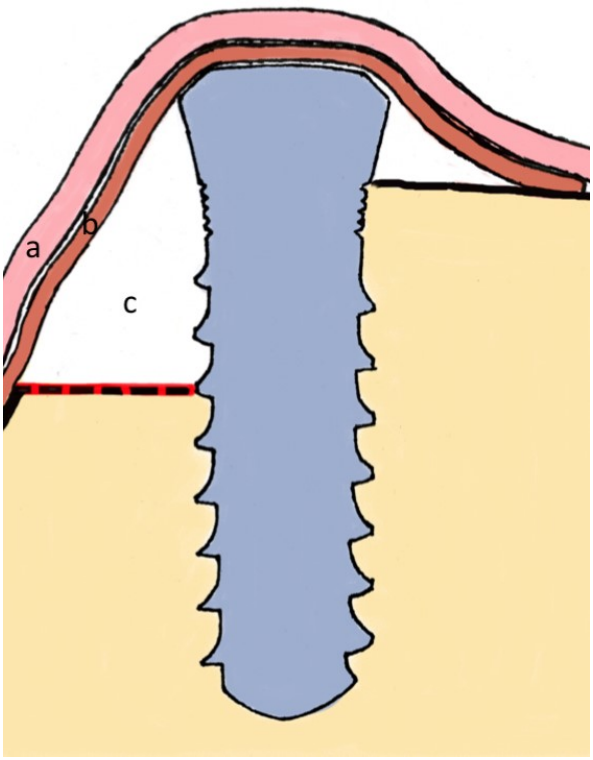

Fig. 7.5: Scheme of empty Part A defects: a: mucosa; b: collagen membrane; c: empty defects; dotted line: cutting line. ***(Illustration Matthias Haab)***

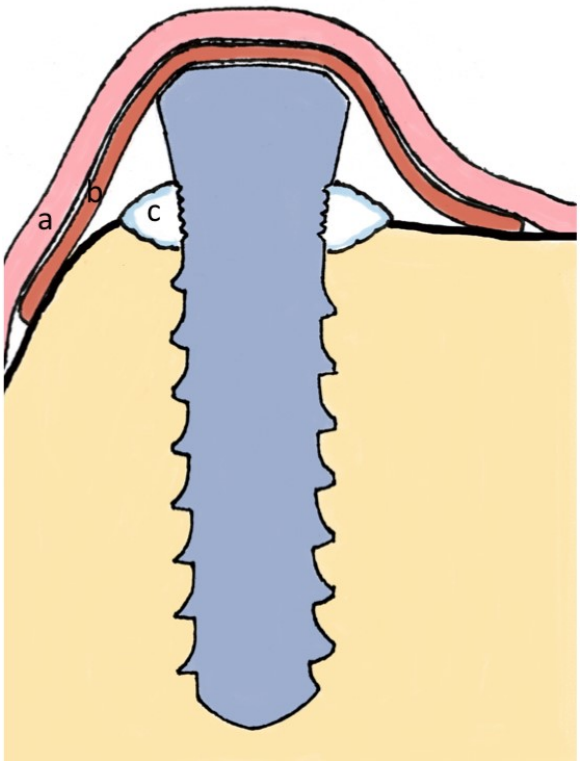

Fig. 7.6: Scheme of filled Part B defects: a: closed mucosa; b: collagen membrane; c: TI ½. ***(Illustration Matthias Haab)***

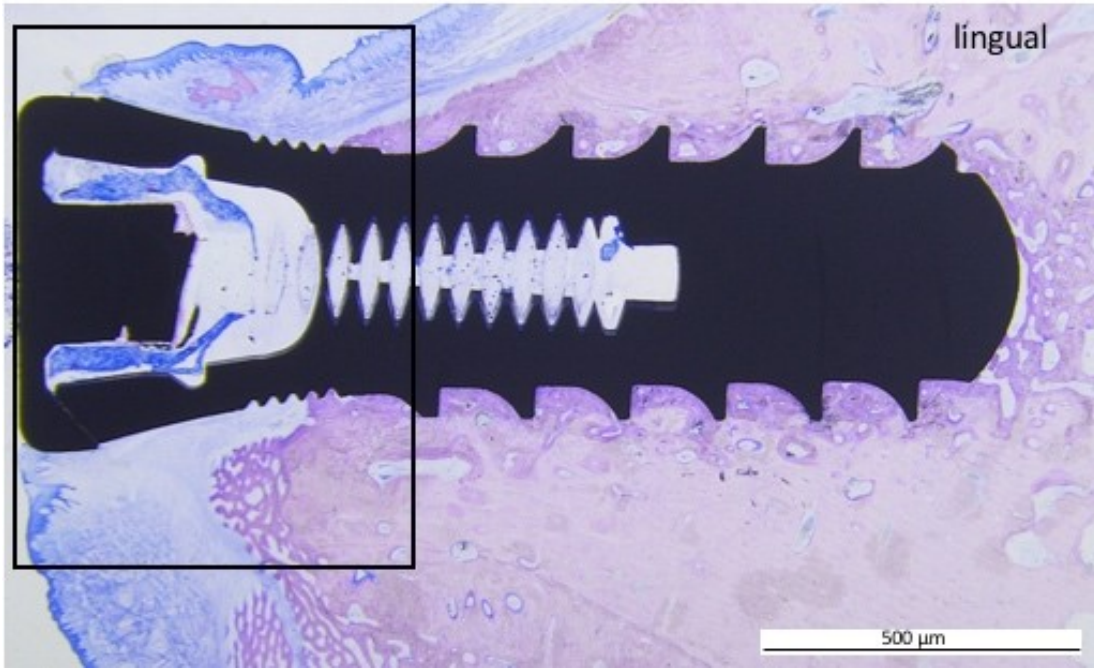

Fig. 7.7: The black box indicates the evaluated area in the histologic evaluation. Magnification: 7.11 x 1.0; Sanderson's RBS

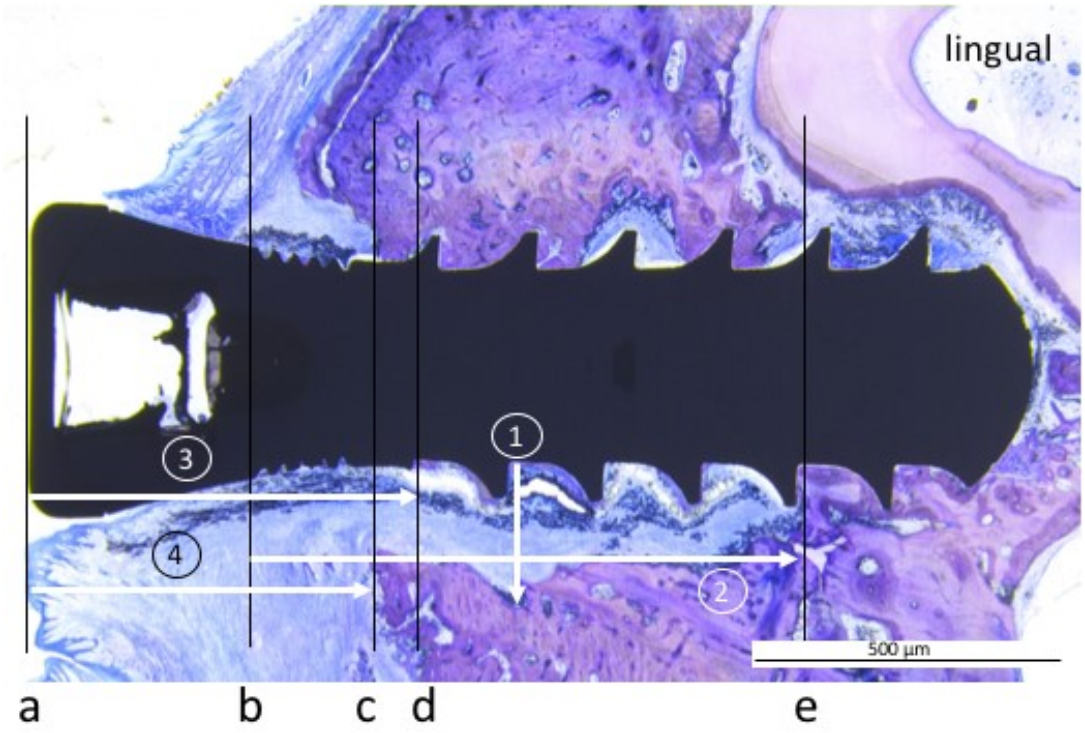

Fig. 7.8: Histologic image (7.11 x 1.0): a: highest point of soft tissue; b: highest of the small threads; c: highest point of bone on the buccal aspect (bone crest); d: deepest point of epithelium ingrowth; e: deepest point of pocket 1: pocket width: measured at the widest point; 2: pocket depth: (distance b – e) – (distance b – c); 3: epithelium invasion: distance a - d; 4: soft tissue thickness: distance a - c.
All measurements in µm. Magnification: 7.11 x 1.0; Sanderson's RBS

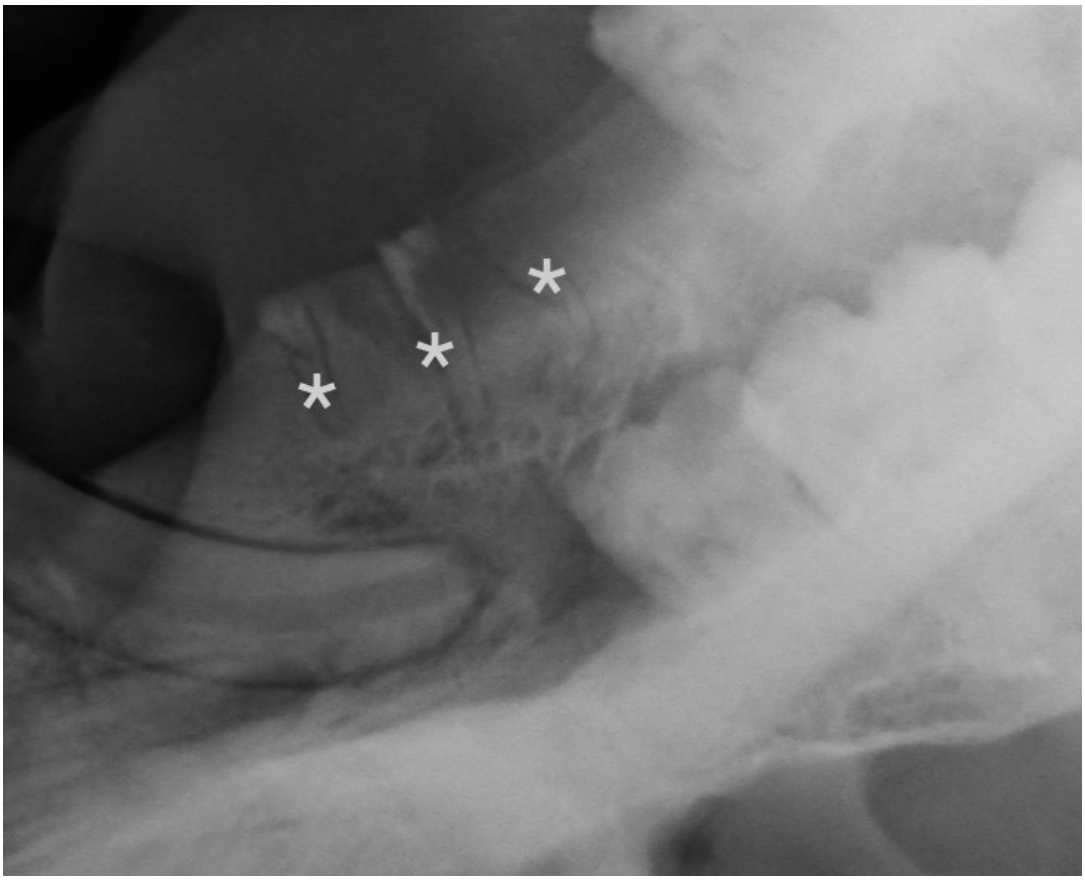

Fig. 7.9: Animal 85.05: Lateral oblique projection of the right mandible. Stars indicate the remnants of the roots of the premolar teeth.

Fig. 7.10: The picture was taken during the third surgery of Part B (debridement and TI application). The sutures around the abutments were in place for 4 weeks. A biofilm and food rests covering the abutments can be seen.

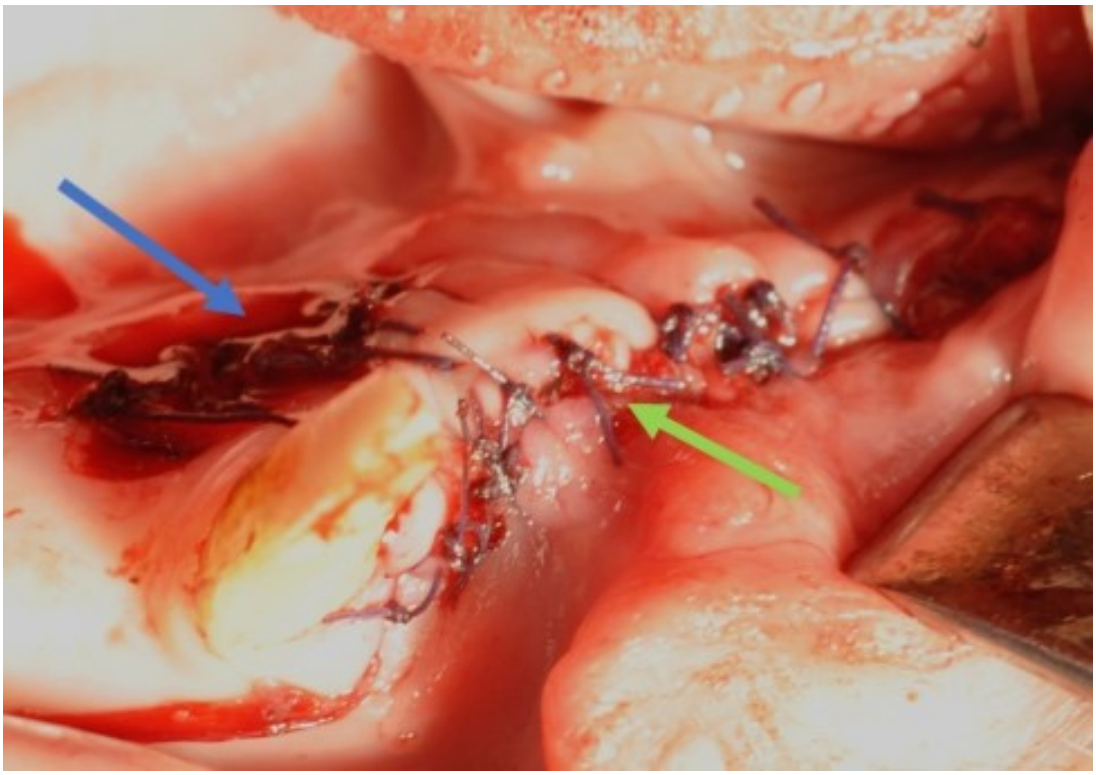

Fig. 7.11: Animal 85.08 after sublingual flap. Sublingual mucosal tissue (blue arrow) had to be mobilized, rotated and closed on top of the implants (green arrow).

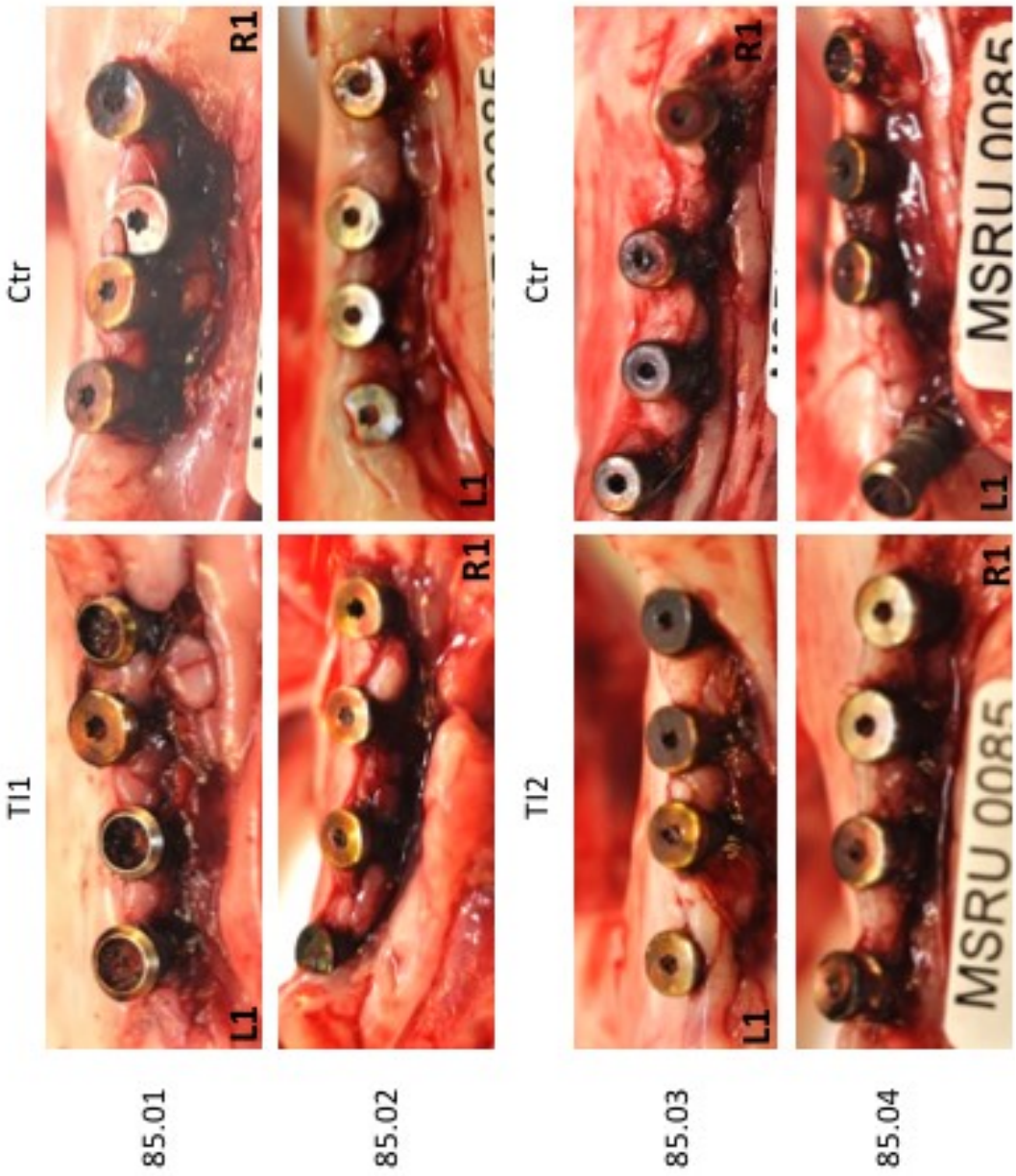

Fig. 7.12: Part A: Pictures were taken after sacrifice and after removal of food rests. Number 1 implants are indicated. Implants are not or only half covered with mucosa. Reddening of the mucosa around all implants is visible.

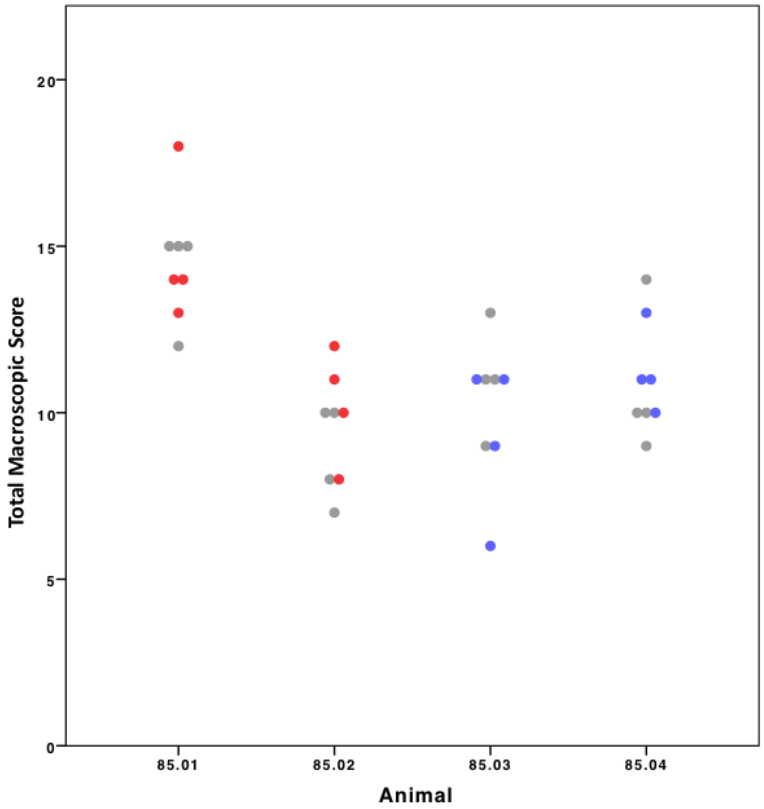

Fig. 7.13: Total macroscopic score (0-21; 0 being the best score) for each implant of Part A. Grey dots: Ctr; red dots: TI1; blue dots: TI2

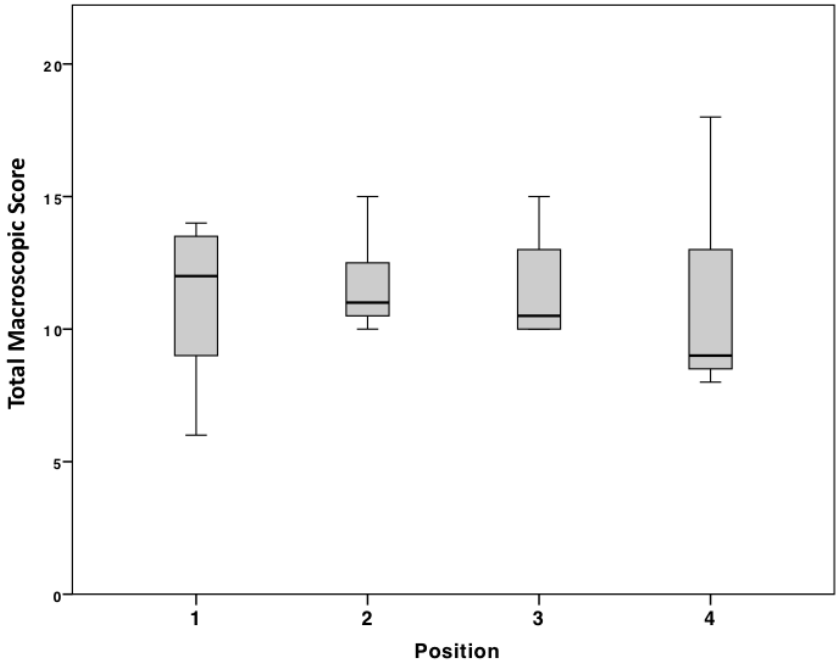

Fig. 7.14: Plot of Total macroscopic score (0-21; 0 being the best score) for positions 1 to 4 of Part A

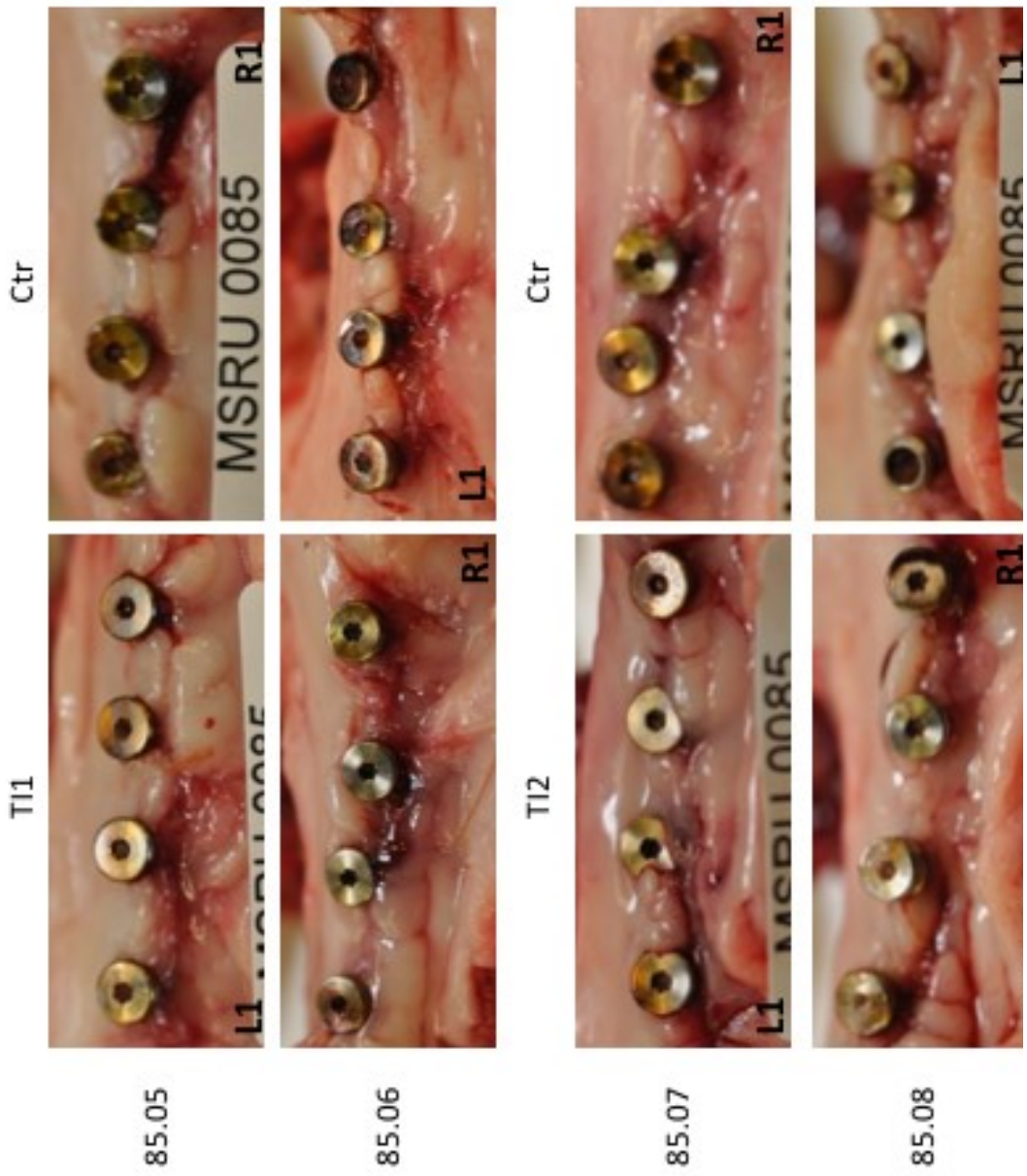

Fig. 7.15: Part B: Pictures were taken after sacrifice and after removal of food rests. Number 1 implants are indicated. Implants are not as exposed as in Part A. Reddening of the mucosa around all implants is visible, but less compared to Part A.

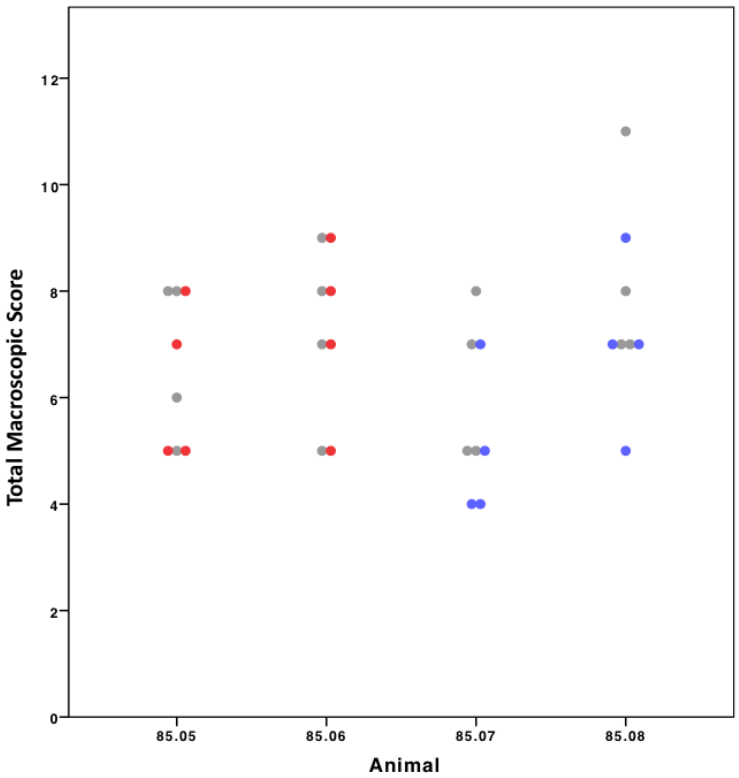

Fig. 7.16: Total macroscopic score (0-21; 0 being the best score) for each implant of Part B. Grey dots: Ctr; red dots: TI1; blue dots: TI2

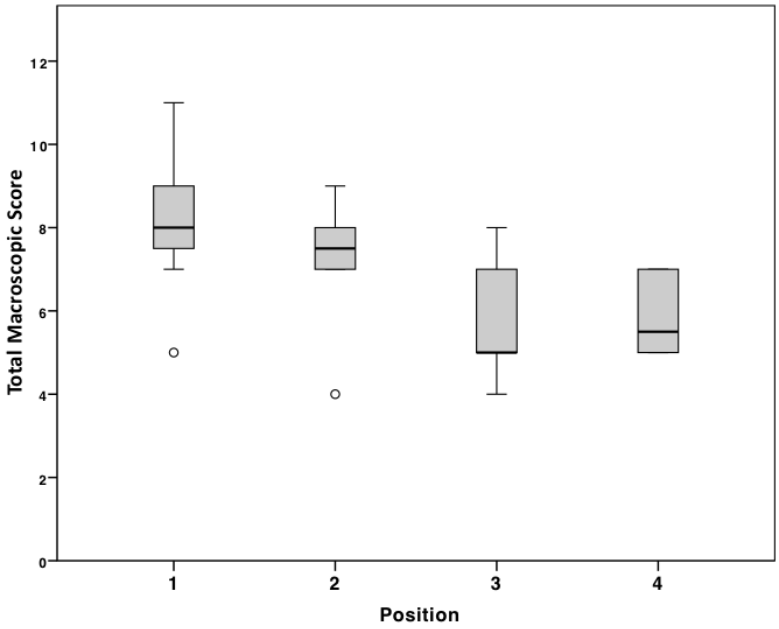

Fig. 7.17: Plot of Total macroscopic score (0-21; 0 being the best score) for positions 1 to 4 of Part B
o position 1: 85.07 R1
o position 2: 85.07 L2

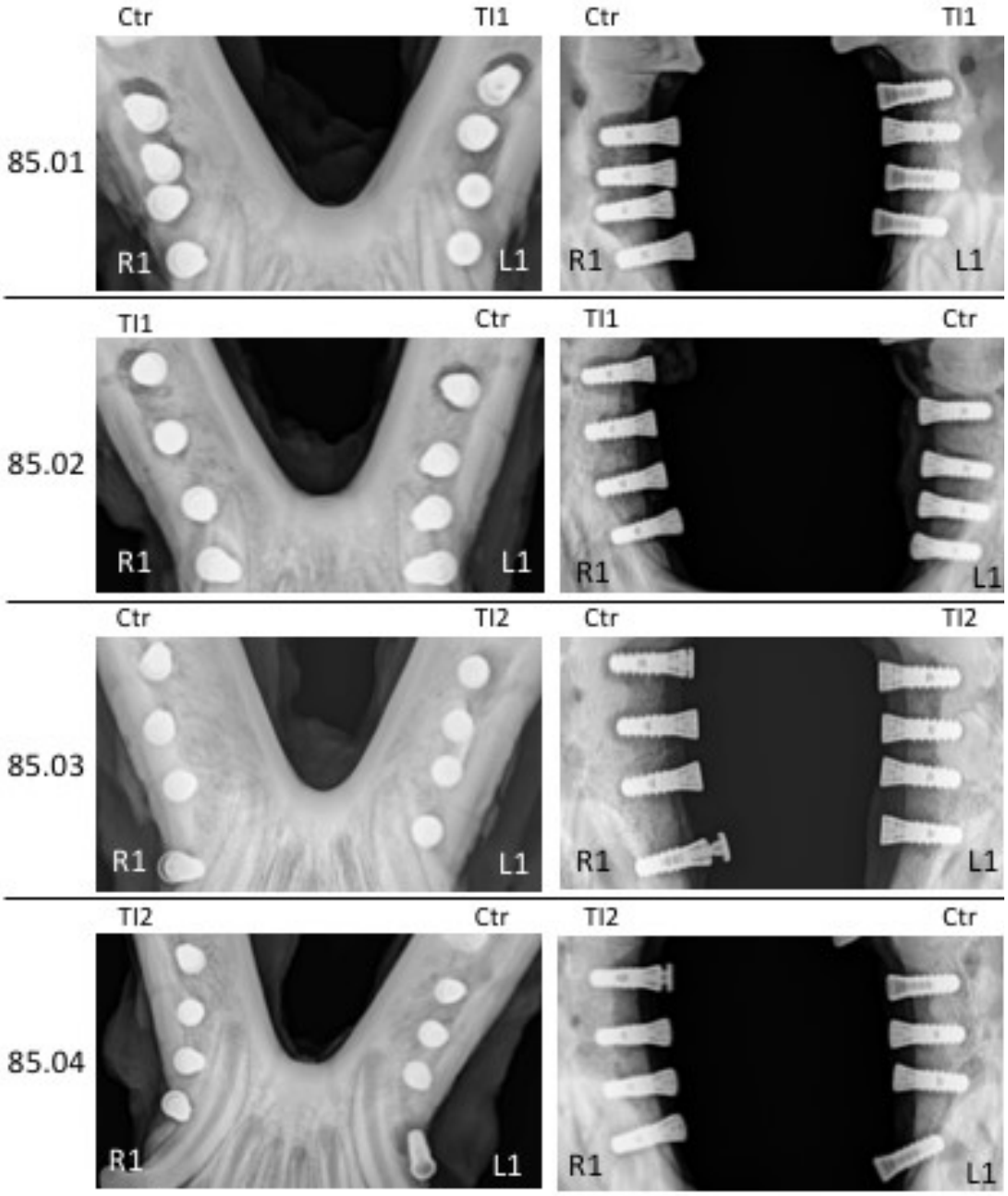

Fig. 7.18: Radiographs of Part A. Dorso-ventral projection on the left pictures and bucco-lingual projection on the right pictures. Position 1 is always indicated.

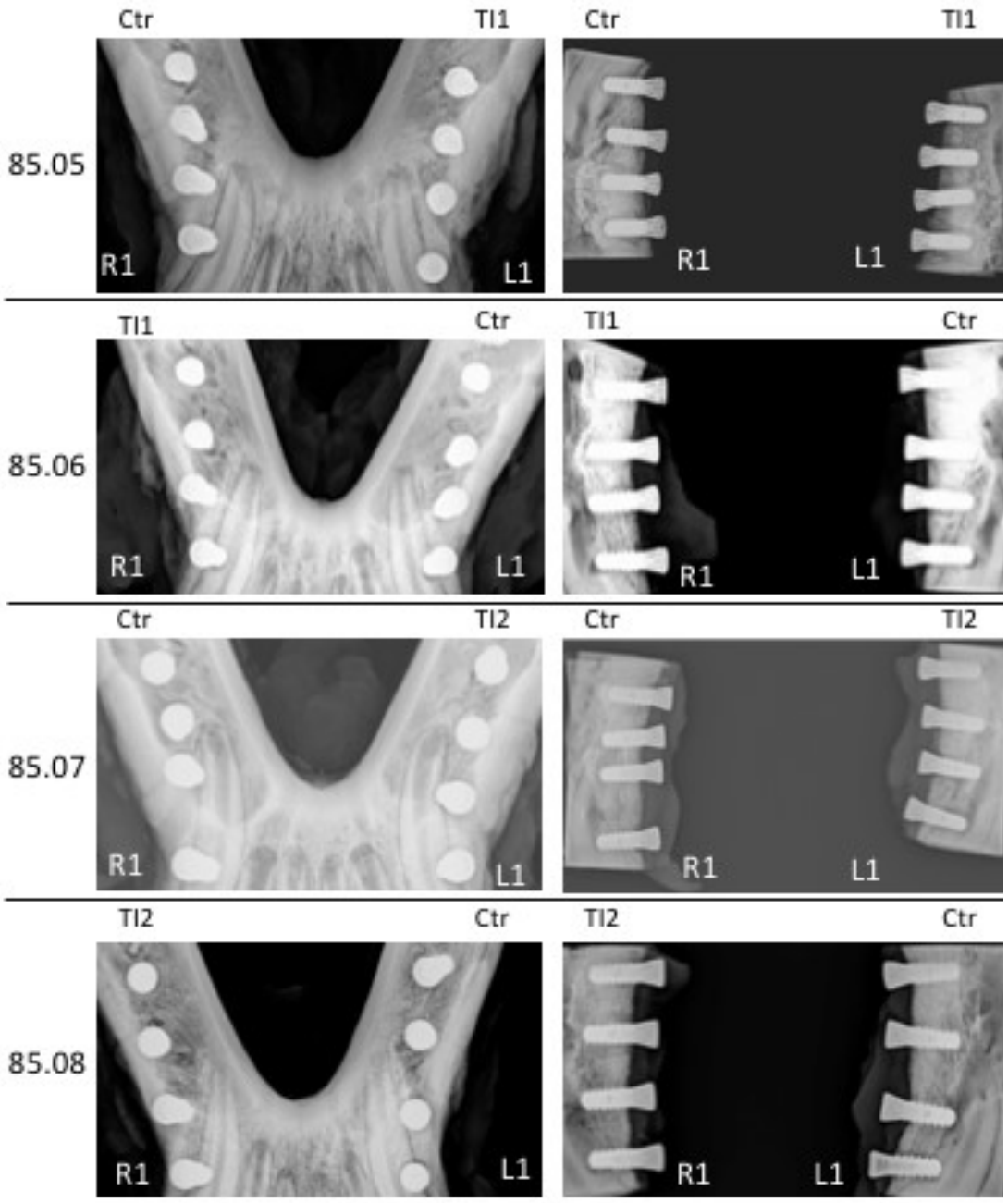

Fig. 7.19: Radiographs of Part A. Dorso-ventral projection on the left pictures and bucco-lingual projection on the right pictures. Position 1 is always indicated.

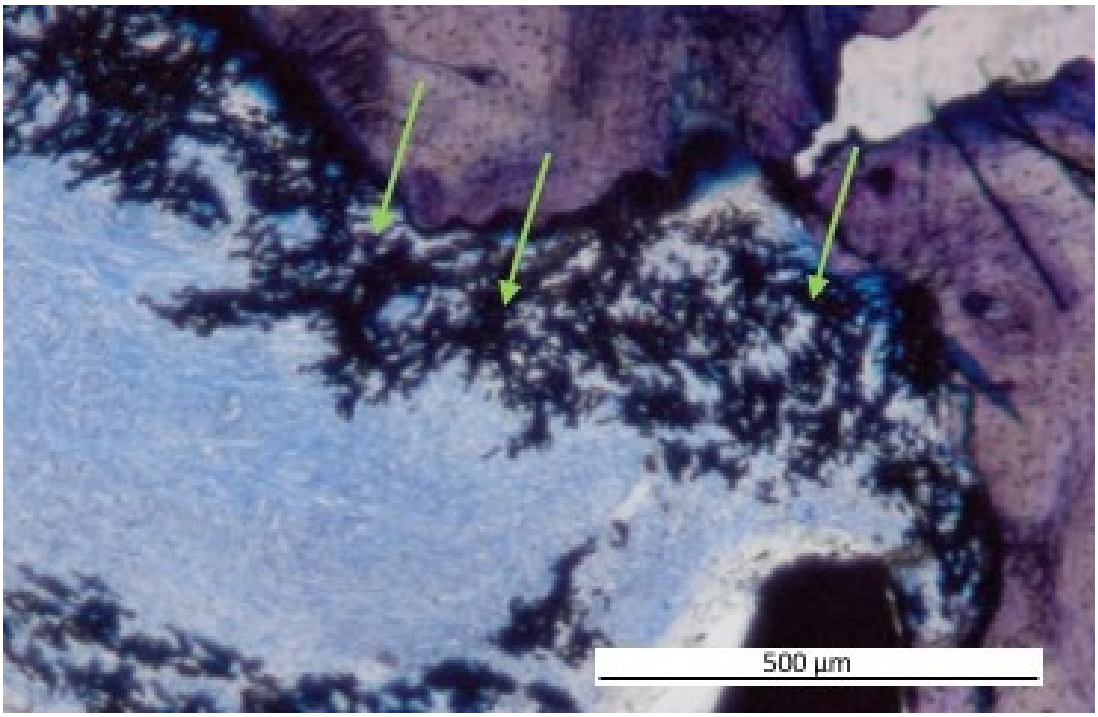

Fig. 7.20: 85.07 L2: black particles are visible within the soft tissue. It is believed, that these particles derived from the implants during the grinding process.
Magnification: 5x10; Sanderson's RBS

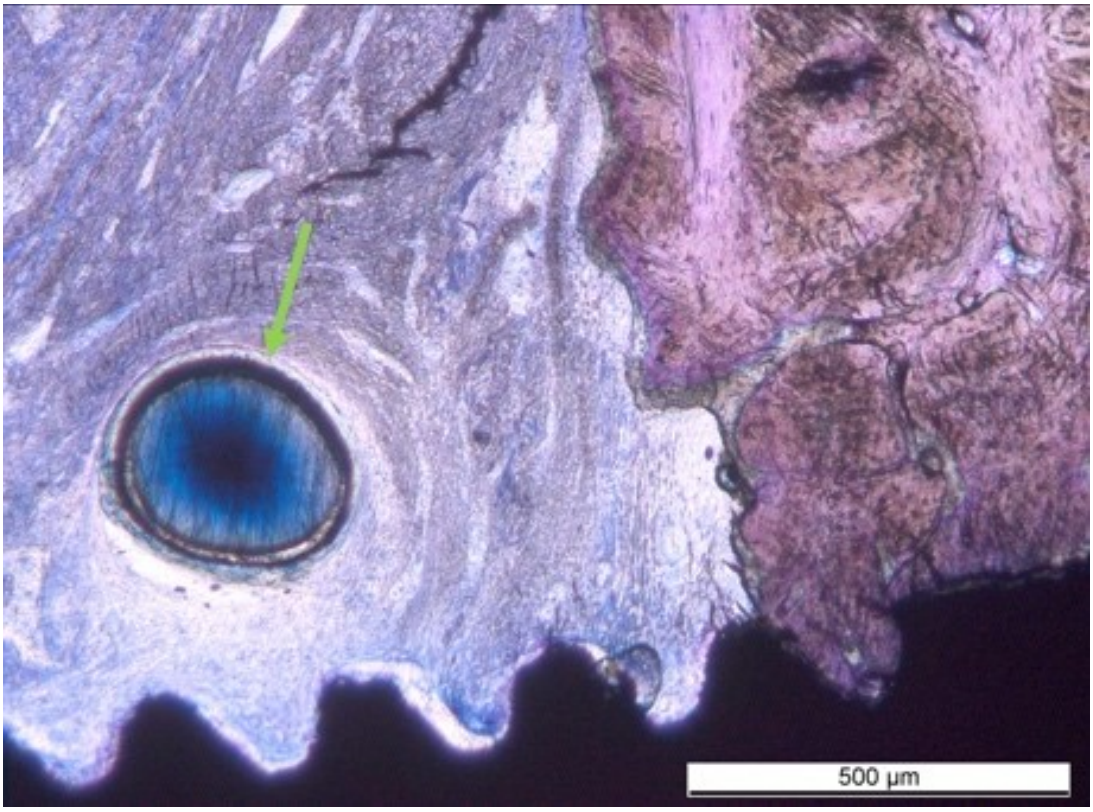

Fig. 7.21: 85.06 L3: the round structure within the soft tissue is a possible remnant of suture material.
Magnification: 5x10; Sanderson's RBS

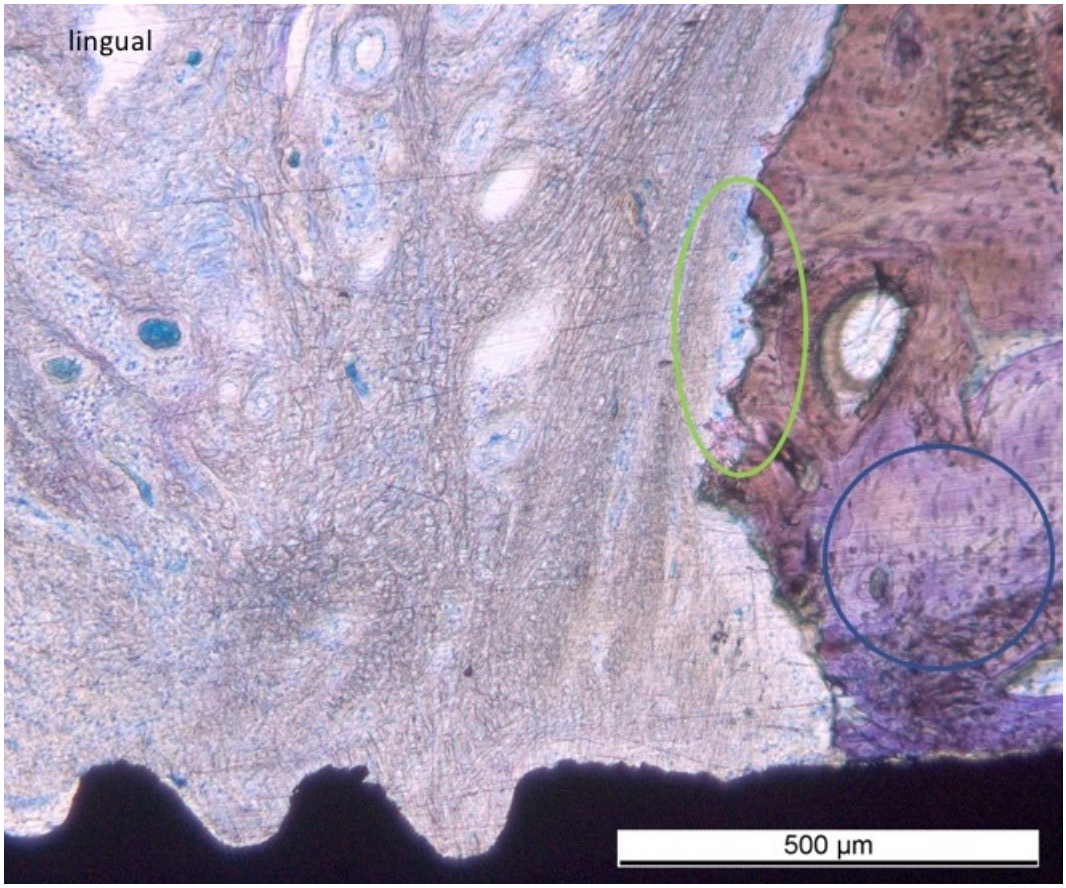

Fig. 7.22: 85.07 L2: A histologic section with resorptive processes (green circle) and young bone (blue circle)
Magnification: 5x10; Sanderson's RBS

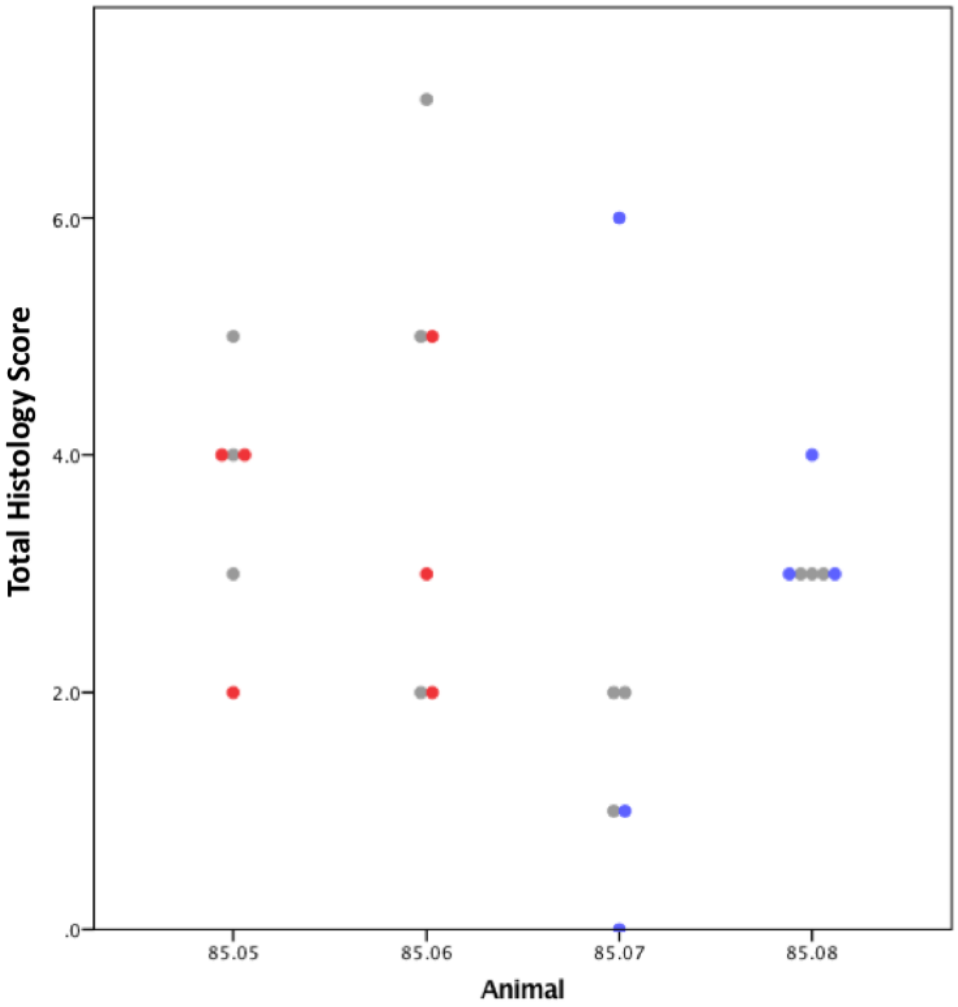

Fig. 7.23: Total Histology Score (0-7; 0 being the best score) of each implant for Part B. Grey dots: Ctr; red dots: TI1; blue dots: TI2

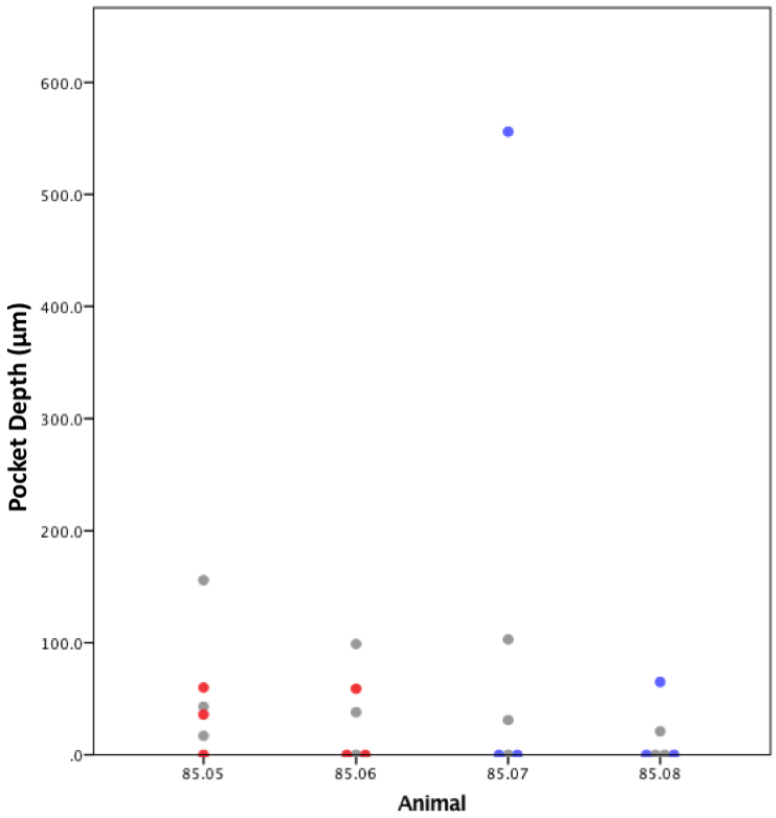

Fig. 7.24: Scatterplot shows the pocket depth of each implant of Part B in µm.
Grey dots: Ctr; red dots: TI1; blue dots: TI2
One outlier is visible: 85.07 L2

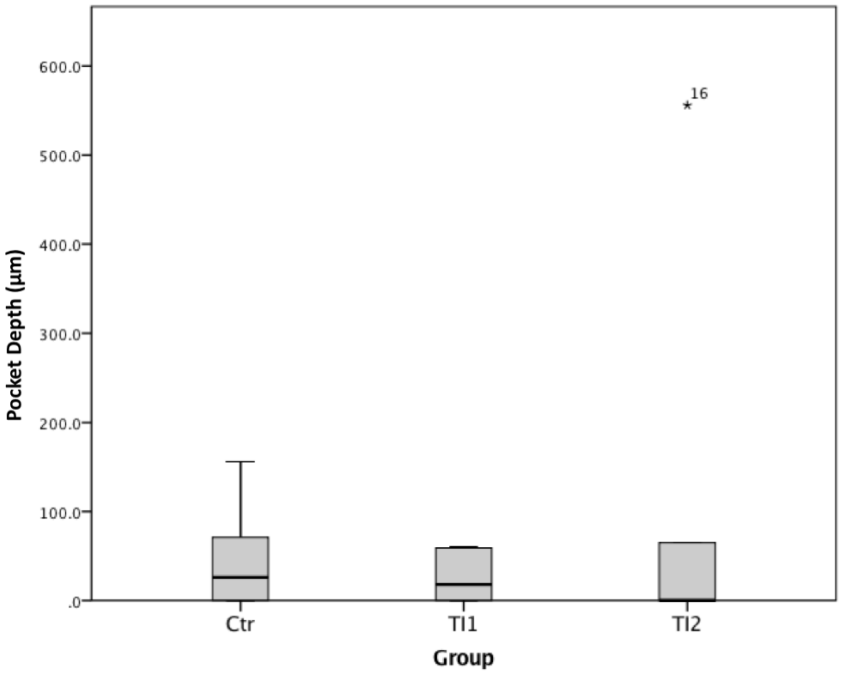

Fig. 7.25: Plot shows the pocket depth in the different groups of Part B in µm.
∗[16]: 85.07 L2, TI2: Outlier with a pocket depth of 556 µm.

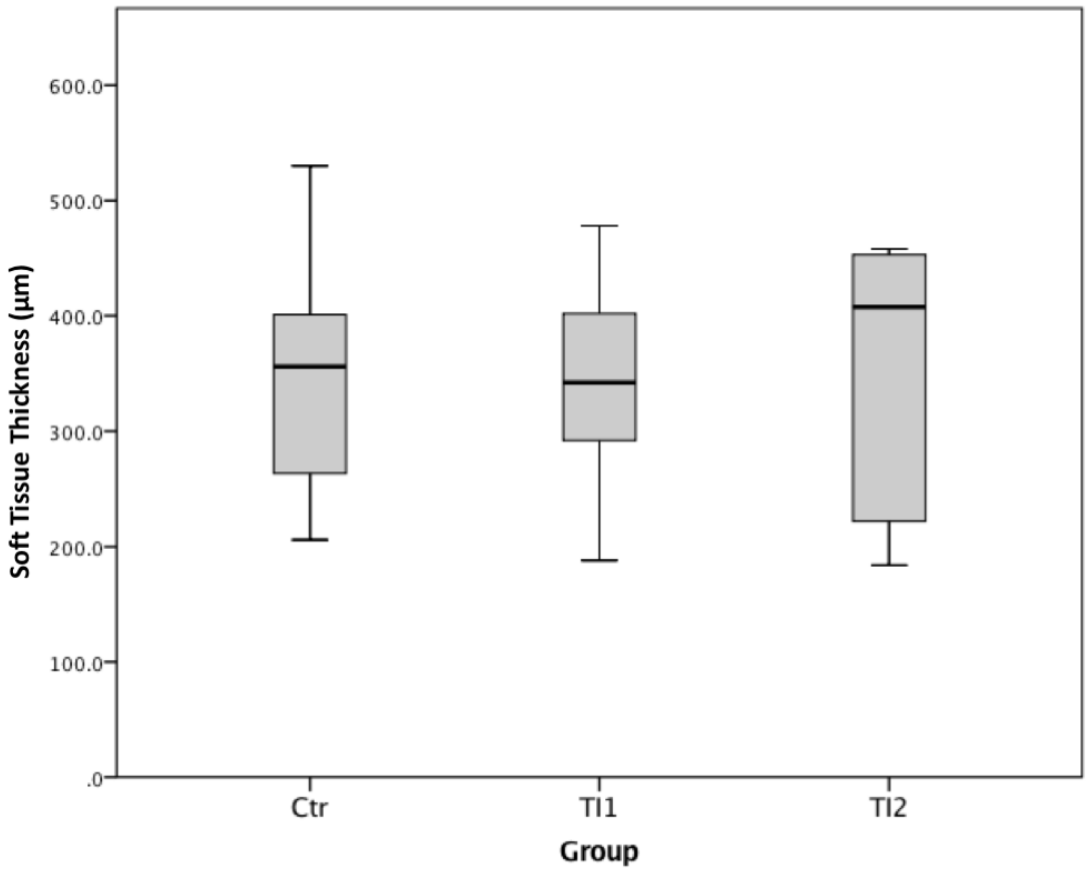

Fig. 7.26: Plot shows the thickness of the soft tissue in the different groups in Part B in µm. All are close together and no big differences can be seen

Acknowledgement

First of all, I gratefully thank Prof. Dr. med. vet Brigitte von Rechenberg for allocating the topic to me and, furthermore, being an inspiring person as a team leader and for her lifework. Right next to her, I would like to thank Dr. Salim Darwiche, for his constantly great supervision and his patience, when I was mixing up something or needed more time than expected for the corrections.

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